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Sodium Ion Transport Regulation by a Serotonin Stimulated Adenylate Cyclase System in Freshwater Mussels.

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ABSTRACT

Freshwater bivalves increase Na influx when the mussels were injected with dibutyryl cAMP, serotonin (5-HT), or 5-HT (10^{-4} M/l) was added to the bath; a response similar to a sodium depleted mussel. Serotonin stimulated Na influx kinetics (K_m = 0.09 mM/l and J_{max} = 4.33 ueq (g dry tissue : hr)$^{-1}$) were similar to Na depleted mussels. Stimulated Na influx (by dibutyryl cAMP or 5-HT) was inhibited by amiloride (5 X 10^{-4} M/l) and the amiloride inhibition was reversible. Serotonin also changes water balance; the 5-HT stimulated mussels gain 0.36 ml H_2O (g dry tissue : hr)$^{-1}$ above controls. Serotonin stimulation of the Na influx was observed to be dose dependent and the net Na flux was related to endogenous gill cAMP content. Cyclic AMP in isolated gills incubated in pondwater containing 10^{-4} M/l 5-HT was highest by 1 min, then declined over 20 min, however the elevated cAMP content was maintained constant with 1.0 mM/l theophylline included in the pondwater. Isolated gills cAMP content increased with the addition of 5-HT, dopamine, norepinephrine and epinephrine (10^{-4} M/l) in pondwater. Adenylate cyclase (AC) activity was present in freshwater bivalve gill tissue and was stimulated by 5-HT (half maximal, 3 uM/l) and dopamine (half maximal, 1 uM/l). Cyproheptadine inhibited the 5-HT dependent increase in AC activity and chlorpromazine inhibited dopamine stimulated AC activity. Prostaglandin E_2, an inhibitor of Na transport in mussels, did not affect mussel gill AC activity. Gill

AC activity was variable and could be inversely related to endogenous calcium with the greatest calcium effect on monoamine (5-HT and dopamine) sensitive AC activity. Crude homogenate pellet AC activity was significantly lower than purified pellet. Exogenous calcium (2.5 mM/l) inhibited monoamine stimulated AC activity in the purified pellet about 50%. Phosphodiesterase and nonspecific phosphatase were highest in the supernatant. The data indicate Na regulation in mussels is dependent on a serotonin stimulated cAMP enzyme system.

CHAPTER I

SEROTONIN STIMULATED ADENYLATE CYCLASE IN THE GILL OF A FRESHWATER MUSSEL AND ITS RELATIONSHIP TO SODIUM TRANSPORT

ABSTRACT

The freshwater bivalve, Ligumia subrostrata, exhibited a 2-3 fold increase in the unidirectional influx of sodium when injected with serotonin (5-HT) or dibutyryl cAMP; a response similar to that of an animal depleted of sodium. The serotonin dependent stimulation of the sodium influx was dose dependent. The Na net flux was directly related to the endogenous cAMP concentration observed in the gill tissue, the principal site of sodium transport in freshwater mussels. The majority of gill adenylate cyclase (AC) activity was located in the pellet of crude gill homogenate (basal activity 21.81 pmol (mg protein $\cdot$ 5 min)$^{-1}$), exhibited halide stimulation (up to 10 fold basal activity) and required 0.1 mM GTP. Gill pellet adenylate cyclase was stimulated by serotonin and dopamine, but not L-dopa, octopamine, norepinephrine, or epinephrine. The gill AC exhibited maximally stimulated activity (2-3 x basal) in the presence of serotonin and dopamine, with half-maximal stimulation occurring at 3.0 μM/l for 5-HT and 1.0 μM/l for dopamine. Cyproheptadine, a serotonin antagonist, inhibited the 5-HT dependent increase in adenylate cyclase activity. The dopamine antagonist, chlorpromazine, inhibited dopamine stimulated gill AC activity. Prostaglandin E_2 (0.2 μg/ml) did not modify either basal or 5-HT stimulated adenylate cyclase activity. Gill pellet AC activity from mussels maintained for over 4 weeks in deionized water were not different from pond water acclimated mussels.

INTRODUCTION

A sodium transport regulatory system in Ligumia subrostrata permits this mussel to maintain a sodium steady state in a dilute environment (Dietz, 1978). The primary site of sodium uptake in the mussel has been demonstrated to be the gill (Dietz and Findley, 1980; Dietz and Graves, 1981). When freshwater bivalves are placed in deionized water, the animal loses ions and a stimulation (compared to control) of the sodium and chloride influx is observed upon returning the mussels to a salt containing medium (Murphy and Dietz, 1976). Sodium transport may be independently stimulated by depleting the mussel of sodium (by maintenance in 1.0 mM choline chloride), but the chloride balance is unaffected (Scheide and Dietz, 1982). A circadian rhythm of sodium balance has been observed in freshwater mussels (Graves and Dietz, 1980), with this rhythmic variation in ion regulation corresponding with periods of mussel activity (Salanki and Vero, 1969; McCorkle et al., 1978; Shirley, 1982). Margaritifera hembeli, displayed evidence of a 'handling response', where by handling the animal initiated a high sodium influx with no effect on Cl transport (Dietz, 1979). These observations suggest a specific, fast acting control system for sodium transport in freshwater mussels.

Several neurotransmitter substances have been implicated in the regulation of sodium transport in freshwater mussels (Dietz et al., 1982). Injections of serotonin, dopamine, norepinephrine and

epinephrine each induced a stimulation of the sodium influx in the whole animal, however only serotonin stimulated the sodium influx in the isolated gill preparation. In addition, the injection of dibutyryl cAMP also results in a stimulated influx. Monoamines capable of stimulating the sodium influx have been identified in freshwater bivalve neural tissue (Zs-Nagy, 1967; Hiripi, 1968; 1972). Monoamine transmitters have been observed to regulate adenylate cyclase activity and the concomitant intracellular cAMP levels (see Greengard, 1976; Nathanson, 1977). In lamellibranchs, serotonin and dopamine have been observed to regulate cAMP formation and adenylate cyclase activity in cardiac tissue (Higgins, 1974; Wollemann and S.-Rozsa, 1975).

Evidence is presented in this paper for the presence of a serotonin stimulated adenylate cyclase associated with the regulation of sodium homeostasis in the freshwater bivalve, Ligumia subrostrata.

MATERIALS AND METHODS

ANIMAL MAINTENANCE

Ligumia subrostrata were collected from ponds near Baton Rouge and acclimated to aerated artificial pondwater (Scheide and Dietz, 1982) for at least one week prior to use. Mussels were ion depleted by placing them in deionized water for at least two weeks. For all the data reported, only males were used to avoid the problem of glochidia brooding in the female gills.

MATERIALS

All compounds used in this study, unless otherwise noted, were purchased from Sigma. Radiotracer flux studies utilized ^{22}Na from New England Nuclear. Tritiated cAMP (26 Ci/mM) and a cAMP Assay Kit were purchased from Amersham.

FLUX STUDIES

Ion net flux was determined by placing the mussel in a container of pond water and monitoring the change in the bath ion concentration as a function of time (Graves and Dietz, 1982). Sodium concentrations were determined by flame photometry. The net flux, J_{net}, was normalized to gram dry tissue of the mussel and expressed as μmol (g dry tissue $\cdot$ h)$^{-1}$. The influx, J_{in}, was measured by monitoring the disappearance of isotope (^{22}Na) from the bath and the change in bath sodium concentration (Graves and Dietz, 1982). The efflux, J_{out}, was calculated by subtracting the net flux from the influx.

GILL cAMP CONCENTRATIONS

Ligumia subrostrata were placed in containers and the net flux was determined. Immediately following the last bath sample, the mussels were opened, the left gill removed, blotted, weighed and homogenized in a 50 mM tris + 4 mM EDTA buffer, a cAMP extraction procedure compatible with the Amersham cAMP kit. The homogenate was placed in a boiling water bath for 5 minutes. The denatured

homogenate was centrifuged at 12,000 x g for 20 minutes and the supernatant was assayed for cAMP and the pellet digested in 1.0 N NaOH for protein determination (Lowry et al., 1951).

CYCLIC AMP BINDING PROTEIN

Out dated human blood was obtained from local hospitals and was utilized as a source of binding protein. The purification technique used was modified from Birnbaumer (1980). All work was performed at 2-4°C. Whole blood was washed 4 times with a 0.15 M NaCl and 5.0 mM potassium phosphate buffer, pH 8.0 to remove the white cell layer. The washed cells were lysed in a 5.0 mM potassium phosphate buffer, pH 8.0 and centrifuged at 30,000 x g for 20 minutes. The pellet was washed repeatedly (5-6 times), then transferred to clean centrifuge tubes until all hemoglobin was visually absent. Cell membranes were precipitated in the presence of 0.75 M NH_4CL for 2 hours and centrifuged at 30,000 x g to pellet the membranes. The pellet was resuspended in 1 volume 5 mM phosphate buffer, pH 7.6. This mixture was diluted 1:1 with a solution of 2.5 mM phosphate buffer, pH 7.6, 50% glycerol and 0.04 mg/ml RIA grade BSA. The final mixture was stored at -60°C and was stable for at least 6 months.

The competitive binding assay for quantifying cAMP content utilizes the cAMP binding protein located in the red cell membrane. Each assay tube had a final concentration of 75 mM tris-Cl (pH 7.6), 7.5 mM $MgCl_2$, 0.75 mM EDTA and ^3H-cAMP (14,000 cpm/tube). Binding of cAMP with the binding protein was allowed to reach equilibrium by incubation at 4°C for at least 18 hr. The assay was terminated with

the addition of a suspension of 1% Mallinkrodt charcoal and 0.1% Dextran T-70. The supernatant of a one minute 8,000 x g centrifugation was counted by a liquid scintillation counter. Observed cAMP (adenosine 3',5' cyclic monophosphoric acid) sensitivity was between 0.1 to 10 pmoles per tube.

ADENYLATE CYCLASE ASSAY

Methods employed were similar to those of Robertson and Osborne (1979). Excised mussel gills were homogenized in a ground glass tissue homogenizer for 60 seconds in 5.0 ml of a 50 mM tris-SO_4 buffer pH 7.6. The homogenate was centrifuged at 3000 x g for 20 minutes at 4^oC. Except where noted, the supernatant was discarded and the pellet was washed in 2.0 ml tris buffer, then resuspended in 5.0 ml buffer per g wet tissue.

Final concentrations in the reaction tube were: 50 mM tris-SO_4, pH 7.6, 10 mM $MgSO_4$, 2 mM theophylline, 0.5 mM EDTA, 0.5 mM ATP, 0.1 mM GTP, 2.5 mM creatine phosphate, 2.5 units per tube creatine phosphokinase and various concentrations of other compounds that are indicated in the results. The above reaction mixture is referred to as the sulfate reaction mixture. A chloride based reaction mixture also was utilized in some observations, in which chloride was substituted for sulfate and the solution was titrated to pH 7.6 with the addition of HCl. The final chloride concentration in the chloride buffer adenylate cyclase reaction was 55 mM/l.

The adenylate cyclase (AC) reaction (total volume 200 µl) was initiated with the addition of the crude pellet homogenate mixture

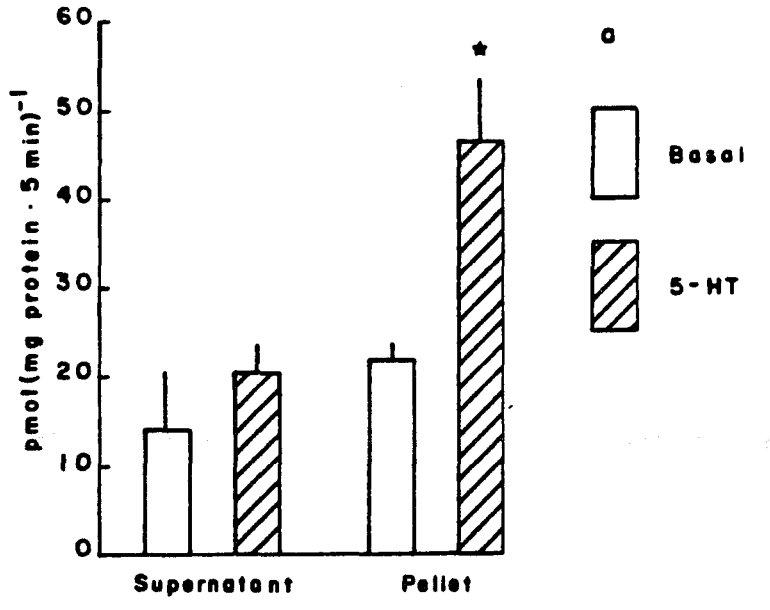
and terminated after 5 minutes with the introduction of 0.8 ml boiling water and the reaction tube was immediately placed in a boiling water bath for 3-5 minutes. The tubes were centrifuged at 7,000 x g for 20 minutes and the cAMP content in the supernatant was determined with the binding protein. The pellet was digested in 1.0 N NaOH and protein content was determined (Lowry et al., 1951). Adenylate cyclase activities are reported as cAMP produced (mg protein · 5 minute interval)$^{-1}$ and "n" refers to the number of gills assayed, with each assay in triplicate.

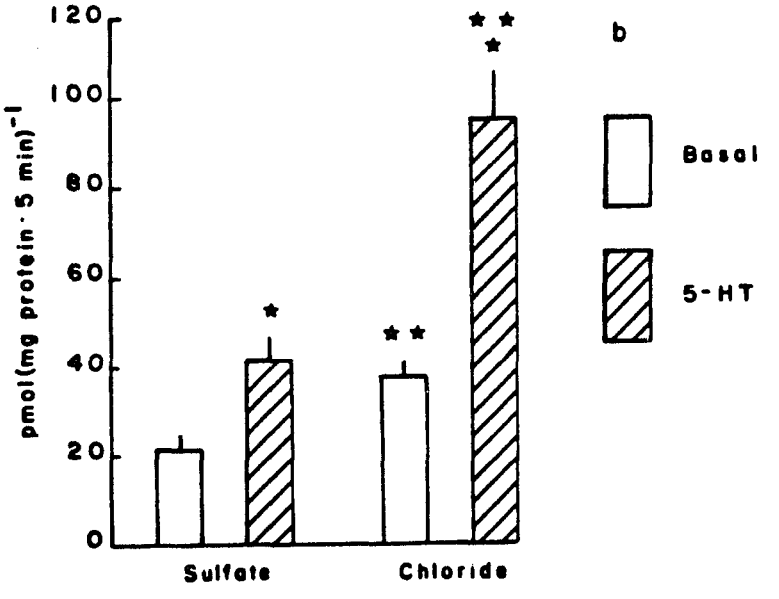
Preliminary results of AC activity indicated a linear reaction for the first 5 minutes of the assay. Maximal AC enzyme activity was dependent upon the presence of 0.5 mM ATP and 0.1 mM GTP. In the absence of GTP, the basal AC activity was the same, however the serotonin stimulated activity was reduced by 32 $\pm$ 2% (based on the mean activity, n=2). Adenylate cyclase activity was primarily located in the pellet, although some activity was present in the supernatant fraction (Fig. 1a). Pellet AC activity was significantly increased ($P < 0.05$) by the addition of serotonin (60 µM/l), more than doubling the activity from 21.8 $\pm$ 2.0 to 46.4 $\pm$ 6.7 pmol cAMP produced (mg protein · 5 min)$^{-1}$ (n=3). The supernatant was observed to be lower in AC activity and was not significantly stimulated by the inclusion of 5-HT (13.9 $\pm$ 6.6 to 20.2 $\pm$ 3.4 pmol cAMP (mg protein · 5 min)$^{-1}$, basal and 5-HT activity, respectively, for n=3 gills).

Two different anion reaction mixtures were used in determining AC activity, a sulfate and a chloride assay mixture, which resulted

Figure 1a. Adenylate cyclase activity in the supernatant and pellet fractions obtained by centrifuging the gill homogenate at 3000 x g for 20 min. The assay was performed in the sulfate reaction mixture. Values represent the mean (n=3) and the lines represent the mean standard error. ★ Significantly different from basal, $P < 0.05$.

Figure 1b. Gill pellet AC activity in sulfate and chloride reaction mixtures. Adenylate cyclase activity is based on 18 gill pellets for the sulfate buffer and 14 gill pellets for the chloride buffer. The vertical lines represent the standard error of the mean. ★ Significantly different from basal in the same buffer, $P < 0.01$, ★★ significantly different from the corresponding condition in the sulfate buffer, $P < 0.01$.


60
50
40
30
20
10
0
pmol(mg protein · 5 min)⁻¹
Supernatant
Pellet
a
Basal
5-HT


120
100
80
60
40
20
0
pmol(mg protein · 5 min)⁻¹
Sulfate
Chloride
b
Basal
5-HT

in different enzymatic activities (Fig. 1b). Basal AC activity in the chloride assay mixture was increased 73% above the sulfate basal activity. Serotonin stimulated enzymatic activity was 127% higher in the chloride assay mixture compared to the sulfate mixture. In a paired comparison, the stimulation of adenylate cyclase by 5-HT in both buffer systems was approximately the same: the chloride buffer 5-HT stimulation was 173 $\pm$ 29% (n=14) above basal activity while 5-HT stimulated enzymatic activity was 145 $\pm$ 38% (n=18) above basal in the sulfate mixture. In addition, gill pellet AC was stimulated by NaF (Fig. 2). The AC activity increased linearly with the NaF concentration ($r=0.85$, $P < 0.001$). Stimulation by NaF exceeded the 5-HT stimulated adenylate cyclase in mussel gills. The cofactor requirements and halide stimulation of cAMP production indicate the enzyme being studied is adenylate cyclase (E. C. 4.6.1.1).

RESULTS

The sodium influx of Ligumia subrostrata was stimulated by several means. Maintenance of mussels in deionized water for 4 weeks causes salt depletion (Scheide and Dietz, 1982). When salt depleted animals were returned to dilute salt solutions, they exhibit an influx more than double that observed in control mussels (Table 1). Stimulation of sodium influx occurred in mussels injected with serotonin (5-hydroxytryptamine) or dibutyryl cAMP (N^6, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid). Serotonin stimulated the influx over 2 fold that observed in control

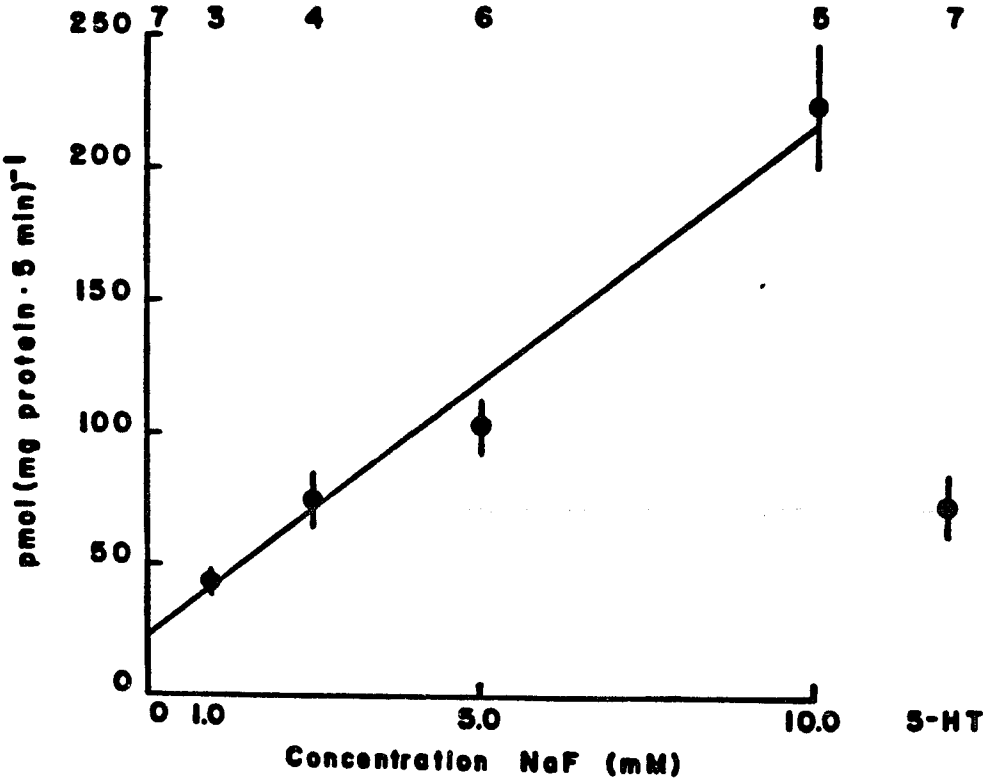


Figure 2. Gill pellet AC activity in the sulfate reaction mixture when the enzyme was incubated with varying concentrations of NaF. The serotonin (60 μM) stimulated AC activity observed with the same tissue is shown on the right side. The line represents values expected from the linear regression (r=0.85). The gill sample size is indicated at the top. Each point represents the mean with the standard error of the mean represented by the vertical line.

Table 1. The effect of ion depletion (DW), the injection of serotonin or dibutyryl cAMP on sodium transport in the freshwater mussel, Ligumia subrostrata, in 0.5 mM Na_2SO_4. Values are expressed as the mean ± the standard error of the mean.

			μmol (g dry tissue · hr)$^{-1}$		
Condition	n	Dose nmol/g dry tissue	J_{in}^{Na}	J_{out}^{Na}	J_{net}^{Na}
Control	41		1.62 ± 0.21	1.31 ± 0.21	0.31 ± 0.28
DW	12		4.10 ± 0.37*	0.78 ± 0.19	3.32 ± 0.29*
Serotonin	10	32 ± 2	3.70 ± 0.56*	1.31 ± 0.28	2.39 ± 0.48*
Dibut. cAMP	18	777 ± 55	5.60 ± 0.46*	1.65 ± 0.36	3.95 ± 0.35*

* Significantly different from control mussels, $P<0.01$.

mussels, while dibutyryl cAMP stimulated the influx 3.5 times the control level. The differences in the two pharmacological treatments were most likely the result of different dosages. The sodium efflux did not vary significantly from control in any of the experimental studies. The stimulated influx resulted in a positive net flux in the experimental groups when compared to the control mussels. In each case, the net flux difference between control and experimental was at least a 700% increase in the inward movement of sodium.

A dose response effect was observed between the sodium transport rate and the amount of 5-HT injected per gram dry tissue (Fig. 3). As the dosage of 5-HT was increased, the influx was observed to increase (semilogarithmic plot, $y = a + b(\ln x)$, $r=0.628$, $P < 0.001$). The maximum sodium influx was about 4 to 7 μmol (g dry tissue $\cdot$ hr)$^{-1}$ when the injected dose of 5-HT was above 40 nmol/g dry tissue.

Endogenous gill cAMP concentrations were related to the net flux of the mussel (Fig. 4). As observed in Table 1, the net flux is an indicator of the sodium transport stimulation. A linear relationship was observed between gill cAMP and the whole animal net flux ($r=0.59$, $P < 0.05$, $n=14$). Mussels in 3 groups were assayed for cAMP: animals acclimated to pondwater, mussels injected with 5-HT (23 ± 2 nmol/g dry tissue) and salt depleted mussels. Group averages for the net flux were: -1.05 ± 0.87 ($n=4$), 1.15 ± 0.36 ($n=5$) and 2.04 ± 0.44 ($n=5$) μmol (g dry tissue $\cdot$ hr)$^{-1}$ for mussels maintained in pondwater, 5-HT injected and salt depleted,

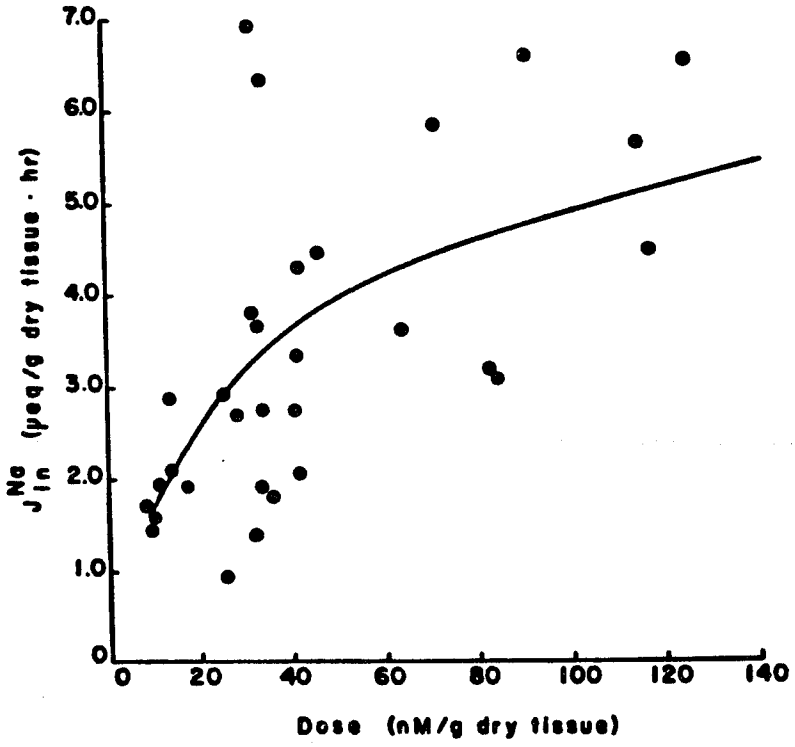


Figure 3. Stimulation of the unidirectional sodium influx, J_{in} in Ligumia subrostrata by injection of various amounts of 5-HT. The solid line represents the predicted value of the sodium influx from the equation, $y = -1.68 + 1.44 (\ln x)$ ($r=0.628$, $P < 0.001$).

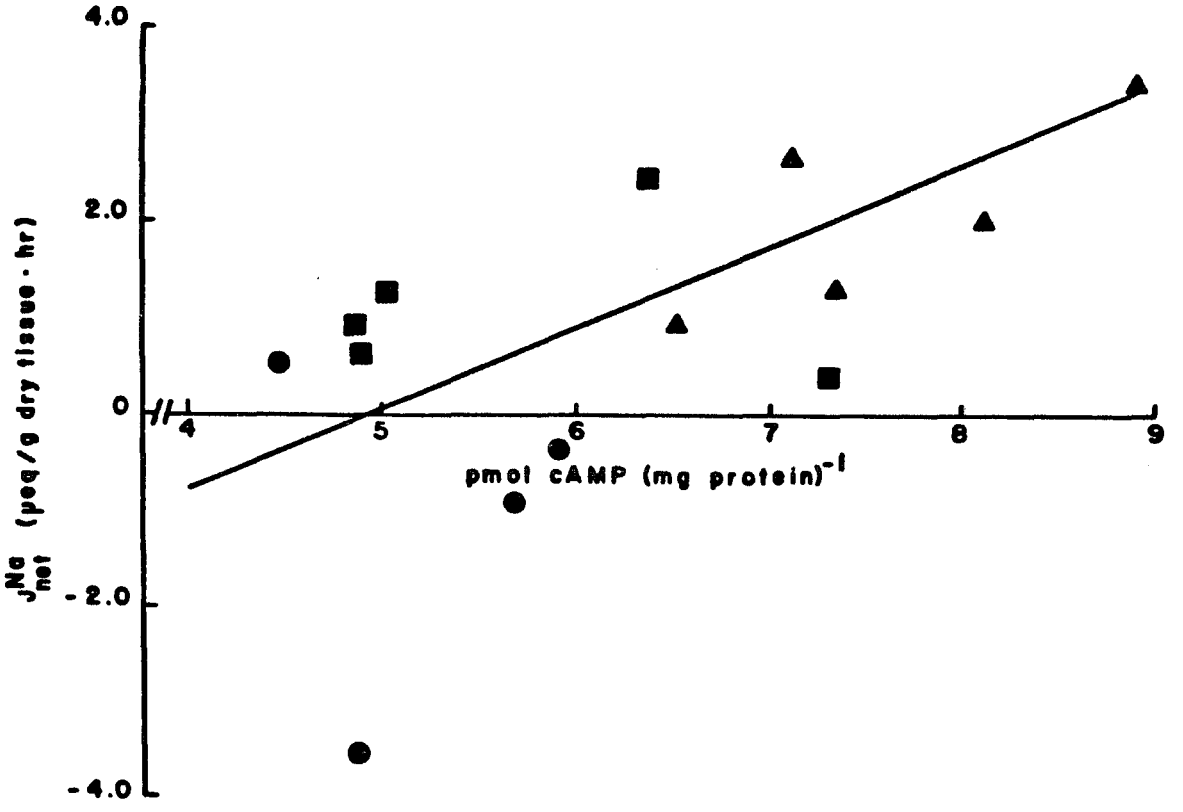


Figure 4. The observed net sodium flux, J_{net}, as a function of endogenous cAMP concentrations in mussel gills. The line represents the linear relationship ($y = -3.82 + 0.75\ (x)$, $r=0.59$, $P < 0.05$). ● = pondwater acclimated mussels; ■ = mussels maintained in DW; ▲ = mussels injected with 5-HT.

respectively. The 5-HT injected and DW-treated mussels experienced a significant increase of the inward movement of sodium ($P < 0.05$). Gill protein and gill wet weight were linearly related ($r=0.92$, $P < 0.001$) and a linear relationship was observed when the net flux was plotted against cAMP expressed as pmol/gram wet tissue ($r=0.55$, $P < 0.05$).

Since adenylate cyclase activity was observed to vary widely from the gills of different animals, the data presented in the remaining figures were paired. Of the various neurotransmitter substances tested, serotonin and dopamine stimulated the AC activity in mussel gills (Fig. 5). In the sulfate based buffer, serotonin significantly stimulated crude homogenate pellet cAMP production ($P < 0.01$), nearly triple the basal activity. Enzyme activity observed in the presence of dopamine averaged twice that of basal however in each gill pellet. Epinephrine, norepinephrine, octopamine and L-dopa did not alter AC activity significantly. All doses were approximately 12 μM. The slight depression of AC activity observed with norepinephrine and epinephrine was consistent, however the enzyme activity could not be further reduced with an increased concentration, up to 0.7 mM/l, of these catacholamines.

Serotonin stimulated AC was concentration dependent (Fig. 6). Pellet AC activity, in the sulfate buffer, was observed to be maximal at 6.0 μM. Increasing the concentration of 5-HT, above 6.0 μM, did not change the serotoninergically related activity, indicating a finite number of serotonin sites activating the adenylate cyclase enzyme. Gill pellet serotonin-activated AC

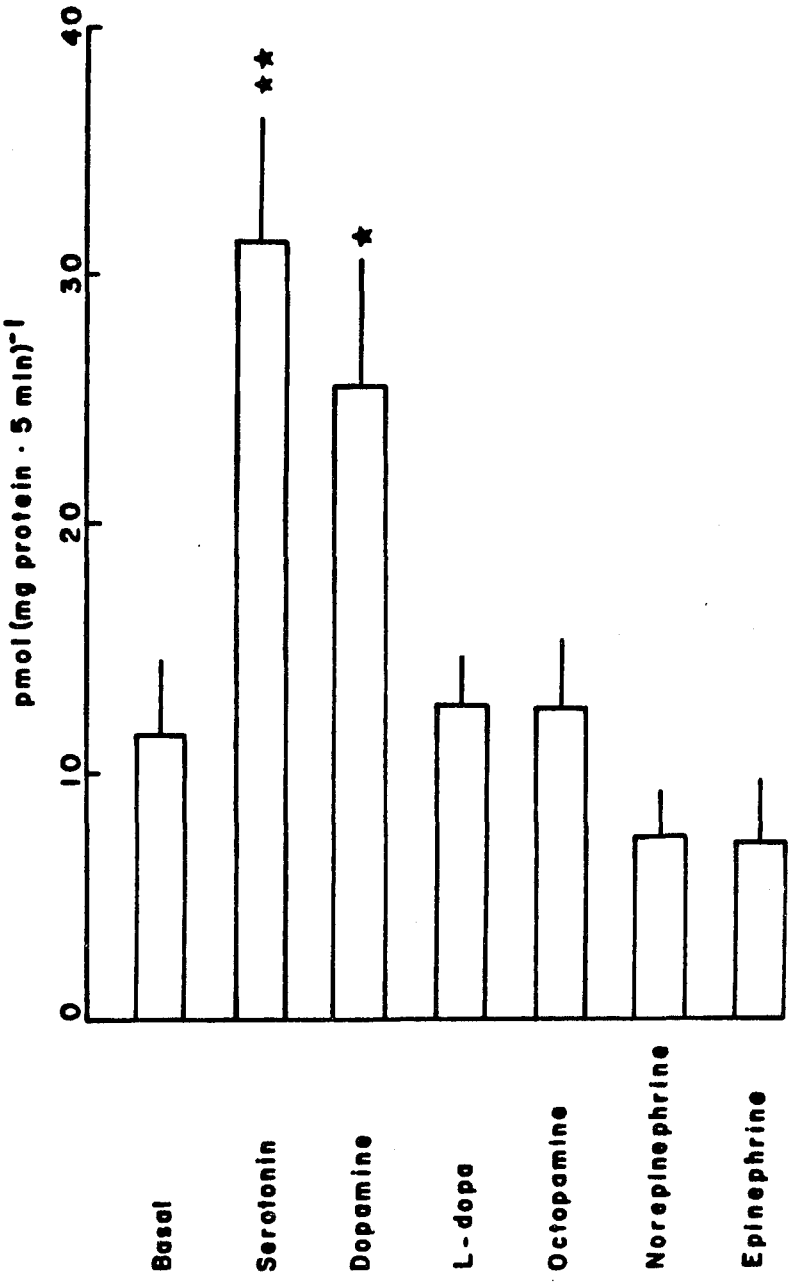


Figure 5. Adenylate cyclase activity observed in the presence of 12 μM of various monoamines in the sulfate reaction mixture. The bars represent the mean value of 3 mussel gill homogenate pellets with the vertical lines the mean standard error. Significantly different from basal activity, ★ $P < 0.05$ and ★★ $P < 0.01$.

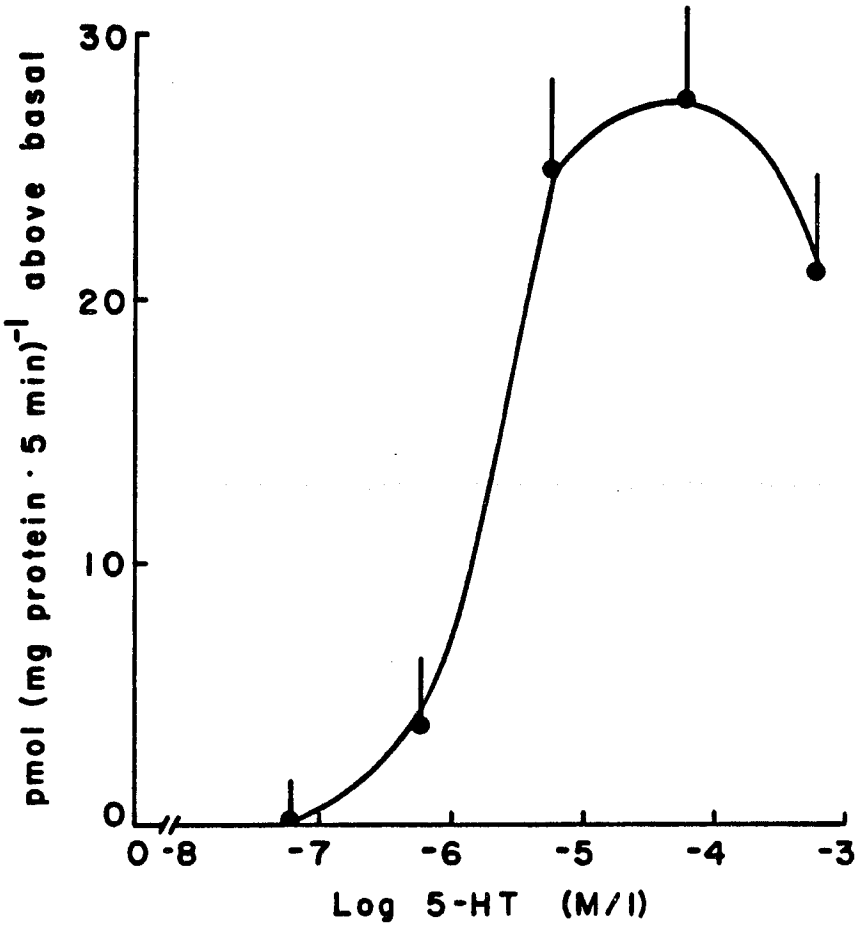


Figure 6. Stimulation above basal of gill pellet AC activity in sulfate reaction mixture incubated in the presence of various concentrations of 5-HT. Basal activity for these observations was 27.0 $\pm$ 0.5 pmol cAMP (mg protein · 5 min)$^{-1}$. Each value represents 3 gills with the vertical lines indicating the standard error of the mean.

displayed a half maximal response around 3.0 μM.

Cyproheptadine has been observed to effectively antagonize serotonin stimulated AC (Nathanson and Greengard, 1974 and Weiss and Drummond, 1981). Cyproheptadine (0.5 mM) abolished the 10 μM 5-HT stimulated activity (Fig. 7). The effects of cyproheptadine blockade of the serotonin activity were evident at 50 μM cyproheptadine ($P < 0.02$). Basal AC activity of 3 gill homogenate pellets was 20.4 ± 1.8, while incubation with 0.1 mM cyproheptadine resulted in a decrease of basal activity to 14.6 ± 1.6 pmol cAMP (mg protein · 5 min)$^{-1}$. A 30% decrease in basal and 5-HT stimulated adenylate cyclase activity was observed, indicating basal activity may, in part, be due to endogenous 5-HT present within the pellet. The calculated K_i from Fig. 7 is 433 μM (with $K_i = IC_{50}$ (1 + c/EC_{50}), K_i is the inhibitor dissociation constant, IC_{50} is the antagonist concentration required to inhibit 50% of the 5-HT activity, c is the serotonin concentration, and EC_{50} is the 5-HT concentration for half maximal stimulation, Weiss and Drummond, 1981).

Dopamine stimulated gill AC activity up to 2 times basal activity, 30.9 ± 1.9 pmol (mg protein · 5 min)$^{-1}$ (Fig. 8). The dopamine stimulated AC activity was saturated above 5 μM with a half maximal response being observed at 1.0 μM. The dopamine response, although significantly greater than basal, did not stimulate gill AC activity to the same degree as 5-HT (Fig. 8 and Table 2). In addition, the two monoamines, when incubated simultaneously, stimulated gill AC activity additively. The increase with both 5-HT

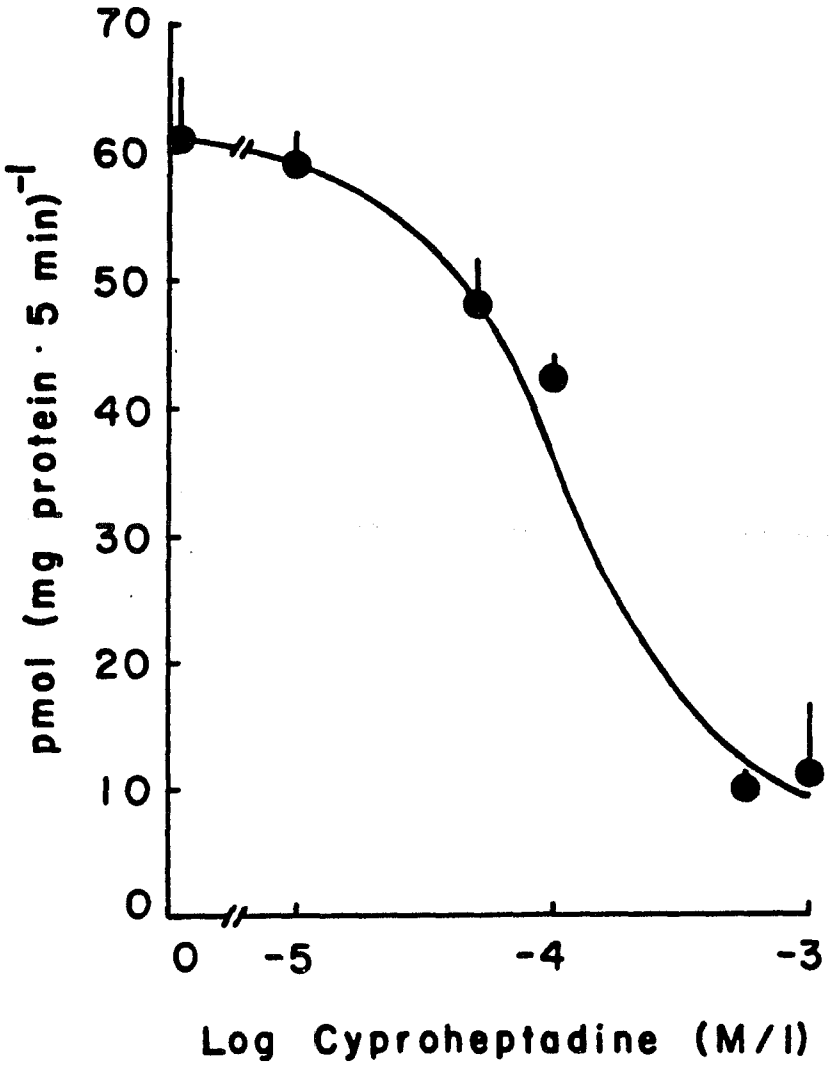


Figure 7. Serotonin stimulated (10 μM) AC activity incubated with various concentrations of cyproheptadine. Each value represents 3 gills with the vertical lines indicating the standard error of the mean.

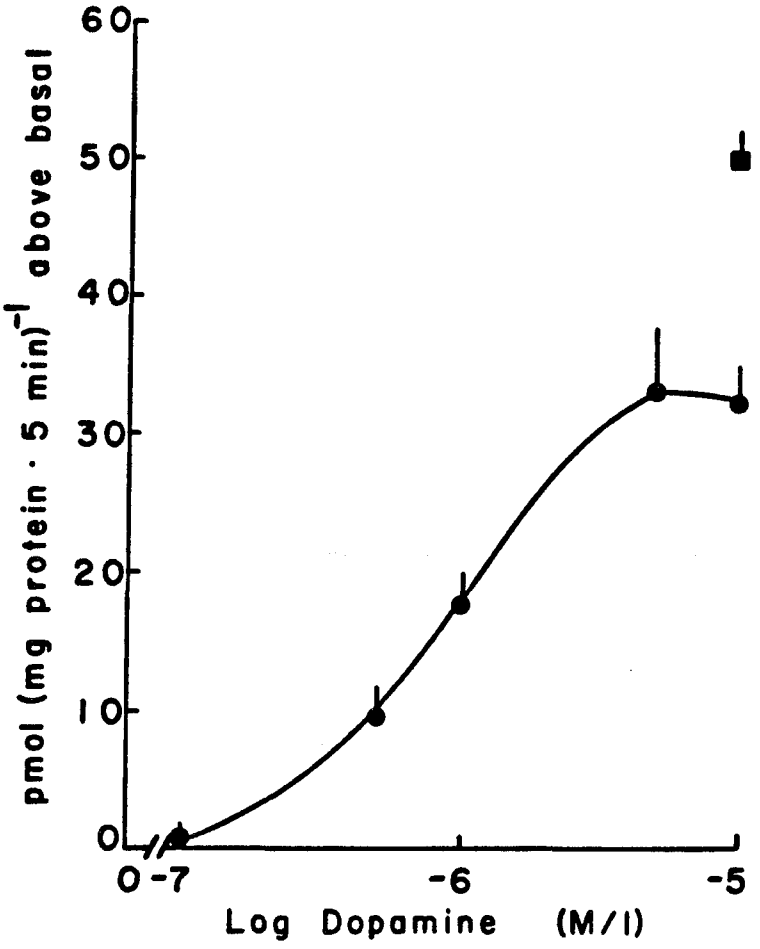


Figure 8. Stimulation of gill AC activity, above basal, when incubated with various concentrations of dopamine. Basal activity was 24.45 $\pm$ 1.54 pmol cAMP (mg protein · 5 min)$^{-1}$ (n=5). Sample size for each point was 3, except for 10 uM (n=5). The square represents 10 μM 5-HT stimulated activity (n=6). Error bars at each point indicate the mean standard error.

Table 2. Adenylate cyclase activity, above basal, when 5-HT, dopamine and both monoamines were incubated with mussel gill homogenate pellet. Basal activity was 20.42 ± 2.44 pmol (mg protein · 5 min)$^{-1}$.

Monoamine(s)	n	pmol (mg protein · 5 min)$^{-1}$ AC activity
5-HT	7	58.19 ± 3.56*
Dopamine	7	43.30 ± 4.06
5-HT and Dopamine	7	72.62 ± 6.01**

* Significantly different from dopamine AC activity, $P < 0.01$.

**Significantly different from 5-HT AC ($P < 0.05$) and dopamine AC ($P < 0.01$) activities.

and dopamine together did not equal the sum of the two monoamines, separately, suggesting some overlap in receptor sites.

Chlorpromazine, an effective dopamine antagonist (Weiss and Drummond, 1981), inhibited ($P < 0.05$) the dopamine stimulated AC activity at 1 μM (Fig. 9). Complete inhibition of the dopamine response was observed at 1 mM chlorpromazine, with 50% inhibition being observed at 2 uM. A K_i of 22 μM was calculcated. Chlorpromazine (0.1 mM) also inhibited basal AC activity by 39%.

Prostaglandin E_2 has been reported to inhibit sodium transport (Graves and Dietz, 1979; 1982; Saintsing et al., 1983). The effect of this fatty acid derivative was investigated on gill AC (Fig. 10). Adenylate cyclase activity of gill homogenate pellet assayed in the presence of 0.2 μg/ml (5.7×10^{-6} M) PGE_2 was similar to basal (18.6 $\pm$ 1.4 and 18.1 $\pm$ 1.6 pmol cAMP (mg protein · 5 min)$^{-1}$, n=5 gill pellets, basal and PGE_2 activity, respectively). The results were the same when PGE_2 concentrations were increased to 50 μg/ml or reduced to 0.2 μg/ml with 5-HT a constant 6.0 μM: the serotonin dependent increase in AC activity was stimulated ($P < 0.001$) over 2.4 times basal (48.9 $\pm$ 4.1, n=5 gills, and 48.1 $\pm$ 5.7, n=4, pmol cAMP (mg protein · 5 min)$^{-1}$, 5-HT and 5-HT + PGE_2, respectively).

Adenylate cyclase activity observed in the gills from mussels maintained for at least 4 weeks in deionized water were similar to mussel gills of animals maintained in pondwater over the same time period. Mussels maintained in DW experienced a positive net flux when returned to pondwater and were significantly different ($P < 0.02$) from control (0.22 $\pm$ 0.08 and 1.96 $\pm$ 0.50 μmol (g dry tissue ·

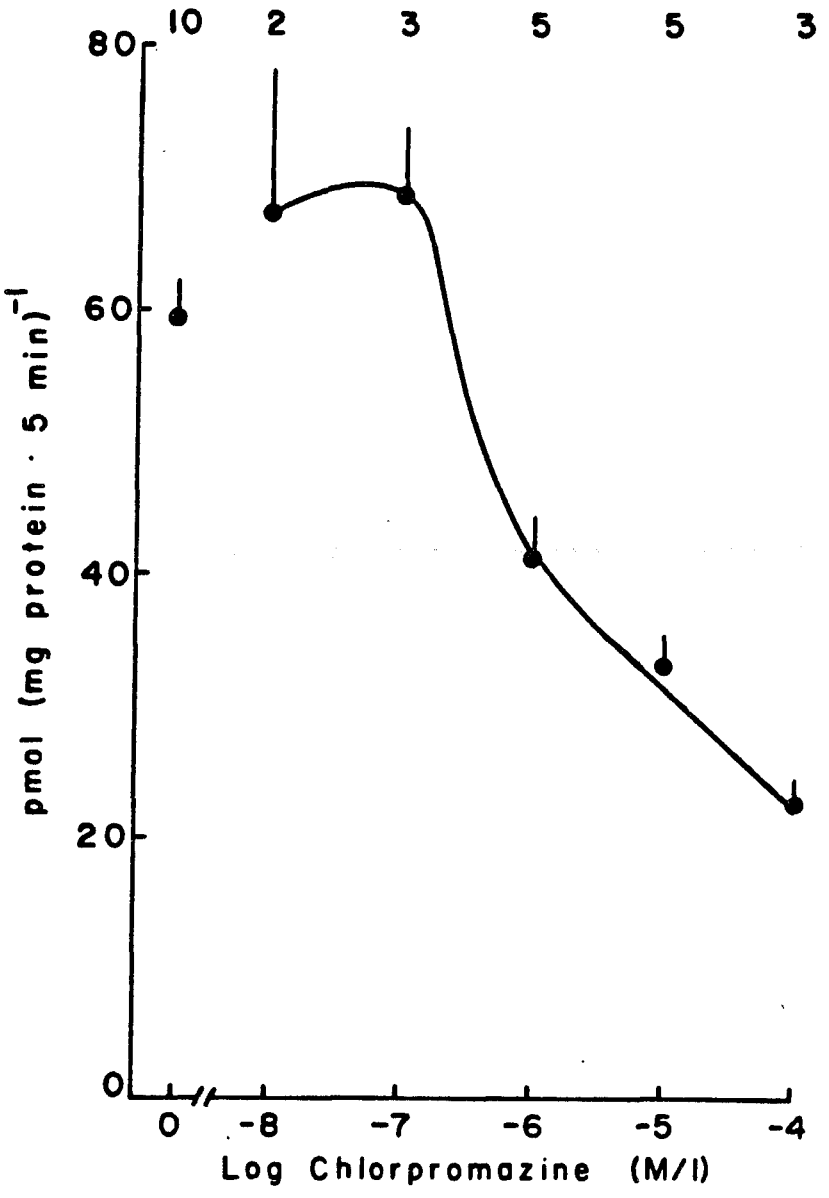


Figure 9. Chlorpromazine inhibition of dopamine stimulated (10 μM) gill AC. Basal AC activity was 25.92 ± 1.41 pmol cAMP (mg protein · 5 min)$^{-1}$ (n=10). The gill number (n) is indicated at the top of the figure. The vertical bars indicate the standard error of the mean.

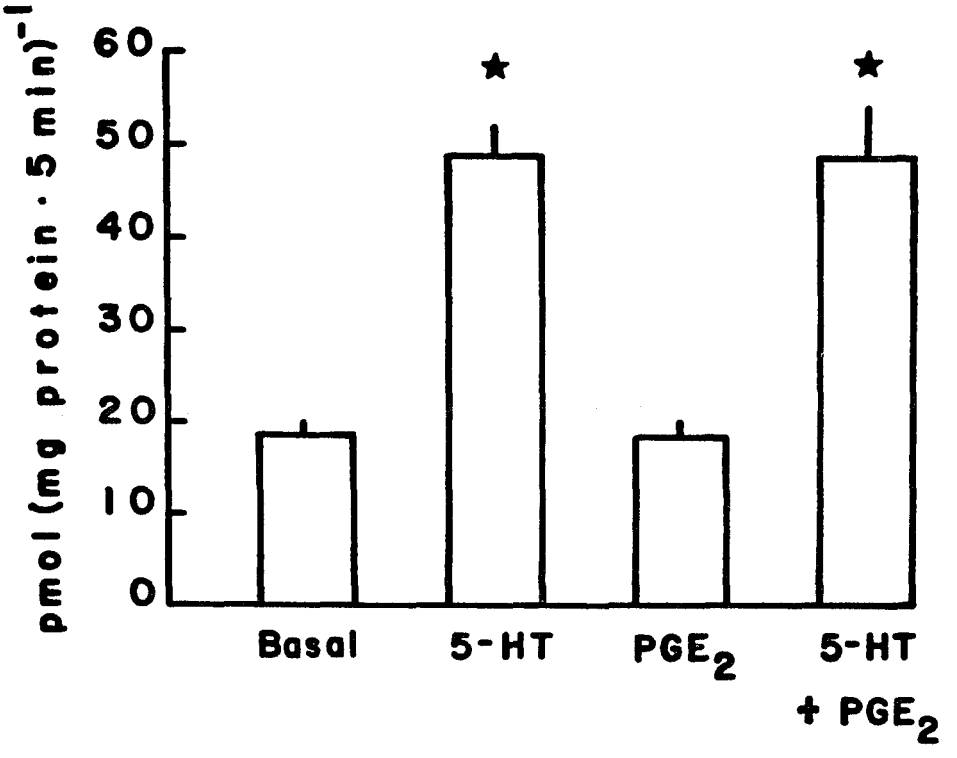


Figure 10. The effects of PGE_2 (0.2 µg/ml) on gill pellet AC in the sulfate reaction mixture. Each value represents 3 or 4 gill pellets. The vertical lines represent the standard error of the mean. ★ Values significantly different than basal and PGE_2, $P < 0.001$.

hr)$^{-1}$ for pondwater and DW mussels, n=4). In the chloride based buffer, basal AC activities were approximately equal (38.1 $\pm$ 3.0 and 38.1 $\pm$ 4.8 pmol cAMP (mg protein · 5 min)$^{-1}$ for pondwater and DW mussel gills, respectively). The serotoninergically stimulated AC activities differed significantly from basal ($P < 0.001$), however they did not differ from one another (81.4 $\pm$ 10.0 and 94.1 $\pm$ 8.8 pmol cAMP (mg protein · 5 min)$^{-1}$ for 4 pondwater and DW mussel gills).

DISCUSSION

Data provides evidence for a serotonin and dopamine stimulated adenylate cyclase (AC) in the crude homogenate of Ligumia subrostrata gills, other monoamines did not alter AC activity. Cyproheptadine inhibited serotonin stimulated AC in gill homogenate pellet and chlorpromazine inhibited dopamine dependent AC. Serotonin stimulated AC activity to a greater extent than that observed for dopamine. The preliminary data indicate there are two populations of receptors, for 5-HT and dopamine, with some overlap in their AC control.

Gill tissue endogenous cAMP content was variable, being highest in salt depleted mussels that had been exposed to pondwater for 2 hours. Injection of a low dose of 5-HT slightly elevated the gill tissue cAMP content from control mussels. The data are consistent with serotonin being responsible for stimulating AC, thus increasing gill cAMP concentrations.

Endogenous gill tissue cAMP content was directly related to the spontaneous sodium net flux. Sodium transport in mussels occurs primarily in the gill and gill sodium uptake is specifically stimulated by serotonin (Dietz, et al., 1982). Both 5-HT and dopamine contribute to the regulation of ciliary motion and both stimulate gill AC, but serotonin is of specific interest for its effect on sodium transport. The injection of either 5-HT or dibutyryl cAMP caused mussels to exhibit characteristics of a sodium-depleted mussel; an elevated sodium influx with no apparent change in the sodium efflux or chloride transport. The injection of theophylline, a phosphodiesterase inhibitor, into freshwater mussels also will elevate the sodium influx above controls (Graves and Dietz, 1982). In the intact mussel, catacholamines (which also stimulate sodium uptake) may act via an adrenergic pathway in the ganglia, presumably the visceral ganglia, mediating the gill serotoninergic mechanism (Dietz et al., 1982).

Ligumia subrostrata gill pellet AC was observed to have many of the characteristics described previously for this enzyme in other preparations. The cofactor GTP was required for serotonin stimulated AC activity in mussel gill as previously reported in Fasciola hepatica (Northup and Mansour, 1978b) and Lumbricus terrestris nervous tissue (Robertson and Osborne, 1979). Adenylate cyclase activity was observed to be halide sensitive, an observation which has been noted by Hynie and Sharp (1971) using NaF and Katz et al.(1980) using NaF and NaCl. The majority of the AC activity in the homogenized gill was observed in the pellet fraction. The cAMP

phosphodiesterase is primarily in the supernatant of the gill homogenate (Hittelman and Butcher, 1972). Although some adenylate cyclase activity was present in the supernatant, phosphodiesterase activity may have been reducing our AC activity measurement.

Gill and nervous tissue AC of Aplysia californica, like Ligumia subrostrata gill, were observed to be sensitive to both serotonin and dopamine (Cedar and Schwartz, 1972; Weiss and Drummond, 1981). The same two biogenic amines were stimulatory to cardiac tissue adenylate cyclase in Mercenaria mercenaria (Higgins, 1974) and Anodonta cygnea (Wollemann and S-Rozsa, 1975). In Ligumia subrostrata gill pellet, the half maximal serotonin stimulation of AC occurred at approximately 3 μM 5-HT, which is higher than that observed in Anodonta cygnea cardiac tissue (0.1 μM, Wollemann and S-Rozsa, 1975) or the thoracic ganglia of Periplaneta americana (0.6 μM, Nathanson and Greengard, 1974), but similar to Aplysia californica gill (1.0 μM, Weiss and Drummond, 1981) and Fasciola hepatica (2.1 μM, Northup and Mansour, 1978a), and less than Lumbricus terrestris neural tissue (20 μM, Robertson and Osborne, 1979). Half-maximal dopaminergic stimulation was at 1 μM, lower than that reported in Aplysia californica gill, 10 μM (Weiss and Drummond, 1981).

The gill of freshwater bivalves has abundant neural connectives coming from the visceral ganglion via the branchial nerve (Splittstosser, 1913). Gill tissue serotonin content has been reported as 0.8 nmol per g wet tissue for Anodonta cygnea, 3.0 nmol per g wet tissue for Unio pictorum (Hiripi, 1968), and 5.8 nmol per

g wet tissue for Ligumia subrostrata (Dietz et al., 1982). Abundant serotonin localized in the gill tissue of freshwater bivalves would be available for the endogenous control of adenylate cyclase.

The inhibition of mussel gill AC by cyproheptadine provides evidence for the involvement of a serotonin receptor coupled to the adenylate cyclase enzyme. The inhibitor dissociation constant calculated for cyproheptadine (K_i=433 μM) was high compared to 0.25 μM for insect thoracic ganglia (Nathanson and Greengard, 1974) or 2.1 μM for Aplysia californica gill (Weiss and Drummond, 1981). A K_i of 22 μM for chlorpromazine inhibition of gill dopaminergic AC is high compared to the 0.75 μM observed in Aplysia californica (Weiss and Drummond, 1981). The differences may be due to AC assay differences, concentration differences or may indicate that these antagonists are not as effective at inhibiting the monoamine stimulated AC activity in mussel gill.

The inhibition of sodium transport by prostaglandin E_2 has been previously observed in freshwater mussels (Graves and Dietz, 1979; 1982; Saintsing et al., 1982). Prostaglandins have been observed to modify ion transport by affecting AC activity (Flores et al., 1975; Iino and Imai, 1978; Schlondorff et al., 1978; Jackson et al., 1980; Stokes, 1981), either by stimulation or inhibition of enzyme activity. In vitro, PGE_2 did not affect mussel gill pellet AC activity at physiological concentrations. Apparently the inhibitory effect of PGE_2 on the sodium influx is not a direct result of modification of gill adenylate cyclase.

The serotonin-cAMP stimulation of sodium transport is similar

to that of the vasopressin-cAMP mediated changes described in the toad bladder (Orloff and Handler, 1962; Hynie and Sharp, 1971; Handler et al.,1979) and kidney collecting tubules (Grantham and Burg, 1966; Imbert et al., 1975). In these preparations direct changes in water permeability and the short circuit current were related to vasopressin, cAMP and theophylline. Opercular chloride transport in Fundulus heteroclitus is regulated by an and B-adrenergic stimulation (Degnan and Zadunaisky, 1979). Vasoactive intestinal peptide and cAMP are involved in the control of chloride excretion in Squalus acanthias rectal gland (Stoff et al., 1979). In the locust, Schistocerca gregaria rectum, a chloride transport stimulating factor-cAMP link has been observed to regulate chloride transport (Spring and Phillips, 1980; Phillips et al., 1980).

Bivalve species inhabiting the freshwater environment must actively regulate their blood ionic concentrations and sodium homeostasis appears to have become a major function of the gills (Dietz and Findley, 1980; Dietz and Graves, 1981). Serotonin is involved in specifically stimulating the influx of sodium in gill tissue with no effect on chloride transport (Dietz et al., 1982). Since sodium regulation in freshwater bivalves is intimately related to a serotoninergically activated system, the range of blood sodium concentrations for the same species reported in the literature may be attributed to different freshwater compositions and/or the neuro-endocrine balance of the animal. Seasonal variation and acclimation temperature related differences of 5-HT levels in ganglia have been noted in aquatic molluscs (Lagerspetz and Tirri,

1968; Stefano and Catapane, 1977; Salanki et al., 1974). The annual rhythms of tissue serotonin concentrations which have been observed may account for the wide range of 5-HT values reported for the same tissue. Seasonal variation of the sodium balance in Anodonta cygnea has been noted with high levels of blood sodium concentrations being recorded in the summer, when tissue 5-HT is high (Nemcsok and Szasz, 1975). Possibly, circadian rhythmicity of serotonin content in nervous tissue may be partially responsible for circadian rhythms of sodium transport observed in the Unionids (Graves and Dietz, 1980; McCorkle, 1982).

Serotonin-cAMP-stimulated sodium uptake may appear to be novel, with a direct neural control of epithelial transport in the mussel. However, freshwater bivalves may be considered to be "primitive", having an open circulatory system and a less structured nervous system (than vertebrates). Thus, neural control of ion uptake may be a more common phenomenon, but, in vertebrates, the epithelial ionic transport functions under neural control may be obscured by changes in blood vessel diameter, blood pressure, and flow, thereby altering the tissue blood supply. It should be noted that serotonin controls many functions in freshwater mussels; cardiac contractions, ciliary motility, valve gape and foot movement. Each of these functions could possibly influence ion accumulation, however the effect of serotonin actually is rather specific in that sodium uptake is stimulated, chloride is not. The freshwater bivalve gill provides an unique model for studying neural control of sodium

transport.

SUMMARY

1. Ligumia subrostrata exhibited a 2-3 fold increase in sodium transport when injected with serotonin (5-HT) or dibutyryl cAMP.
2. The serotonin stimulation of the sodium influx was dose dependent with a maximum sodium influx of 4-7 μmol (g dry tissue · hr)$^{-1}$ with an injected dose of 40 nM 5-HT/ g dry tissue.
3. The net sodium flux was directly related to the endogenous gill cAMP content.
4. Adenylate cyclase activity was greatest in the gill homogenate pellet. Serotonin and dopamine stimulated the AC activity 2-3 fold over basal activity. The gill pellet AC activity was maximally stimulated with 6 μM 5-HT and 5 μM dopamine.
5. Cyproheptadine inhibited the serotonin stimulated AC activity and chlorpromazine inhibited the dopamine stimulated gill AC activity.
6. Adenylate cyclase activity was not modified in the presence of PGE_2, L-dopa, octopamine, norepinephrine or epinephrine. Ion depletion, a process which results in stimulating the sodium influx, did not influence AC activity.

CHAPTER 2

SEROTONIN STIMULATED ADENYLATE CYCLASE IN FRESHWATER MUSSELS AND THE INFLUENCE OF CALCIUM ON ADENYLATE CYCLASE ACTIVITY

ABSTRACT

Sodium influx is rapidly stimulated, as are behavioral responses, when serotonin (5-HT) is added to the pondwater bath (final concentration of 10^{-4} M/l) of steady state freshwater mussels. Serotonin also stimulates adenylate cyclase in gill homogenate pellets of 2 different freshwater bivalve families, indicating that a serotonin-cAMP system is responsible for the stimulation of Na transport and that this system may be present in many ion regulating species. Adenylate cyclase is present in various tissues of Ligumia subrostrata with highest activity being observed in the foot an organ responsible for coordinated muscular activities during burrowing. Endogenous calcium inversely affected basal, 5-HT and dopamine stimulated AC activity with the depressed activity being most pronounced on the monoamine stimulated activity. Crude homogenate pellet AC activity was significantly lower than purified pellet formed by an additional 100 x g centrifugation prior to the 5,000 x g centrifugation. Exogenous calcium (2.5 mM/l) inhibited monoamine stimulated AC activity about 50%. Calcium concretions exist in gill tissue and may influence the observed AC activity by increasing non-functional protein or increasing the free calcium concentration. Prostaglandin E_2 had no effect on basal or 5-HT stimulated AC activities in the purified pellet. Phosphodiesterase and nonspecific phosphatase activities in the homogenate pellet fraction are not enough to be of consequence in

the AC activity measurements, however these activities were high in the supernatant.

INTRODUCTION

Freshwater mussels maintain blood ionic concentrations (Na, Cl, Ca, and HCO_3) above pondwater (Dietz, 1979). Sodium and chloride transport are independent of each other in freshwater mussels (Scheide and Dietz, 1982) with the gill being the primary site of ion accumulation, at least for Na (Dietz and Findley, 1980; Dietz and Graves, 1982). Although the mechanisms by which most of these ions are regulated have not been elucidated, sodium ion regulation appears to be the function of a serotonin coupled adenylate cyclase system in some freshwater bivalves (Dietz et al., 1982; Scheide and Dietz, 1983). A 5-HT dose related effect on sodium influx has been observed and gill cAMP levels are directly related to sodium transport rates, in addition a 5-HT stimulated adenylate cyclase (AC) is present in mussel gill tissue (Scheide and Dietz, 1983).

Ligumia subrostrata gill tissue AC activity was observed to be variable between gill tissues. Adenylate cyclase is a calcium sensitive enzyme. Activity appears to be biphasic with 1-100 μM/l calcium being necessary for AC activity while 0.1 to 1.0 mM/l is inhibitory (Hynie and Sharp, 1971; Bockaert et al., 1972; Brostrom et al., 1977). Calcium could be the source of gill tissue variability due to the presence of calcium concretions in the gill

(Silverman et al., 1983).

In this report, we will investigate sodium transport stimulation by serotonin in several species of freshwater mussels and relate this to the occurrence of 5-HT stimulated adenylate cyclase. We will also survey the tissue occurrence of 5-HT stimulated AC in Ligumia subrostrata. The variability of AC activity between gill tissues will be investigated.

MATERIALS AND METHODS

All organic chemicals were purchased from Sigma Chemical Co. Sodium flux determinations used ^{22}Na purchased from New England Nuclear. The ^{3}H-cAMP (26 Ci/mM) used in the binding protein assay was purchased from Amersham.

Locally collected representative members of the Unionidae, Anodonta grandis, Carunculina texasensis, Ligumia subrostrata and Corbiculidae, Corbicula fluminea, were placed in aerated artificial pond water (0.5 mM/l NaCl, 0.4 mM/l $CaCl_2$, 0.2 mM/l $NaHCO_3$, and 0.05 mM/l KCl). Bivalves were acclimated to laboratory conditions for at least five days prior experimentation. When possible, males were used to avoid interference by the brooding of glochidia.

Blood samples were taken by cardiac puncture (Scheide and Dietz, 1982). Foot ventral edge fluid was collected by holding the wide end of a transfer pipette to that portion of the foot, when an expelled stream of fluid was visible, the pipette was placed directly over the fluid stream. Blood and foot fluid was

centrifuged for 1 min at 8,000 x g and ion analyses used were flame photometry (sodium), electrometric titration (chloride) and atomic absorption (calcium).

Sodium flux analysis was performed to show the immediate effect of serotonin (5-HT) on sodium transport. Bivalves were rinsed for 1 to 2 hours in DW then placed in individual containers of ^{22}Na containing pondwater. After the animals were observed to open and commence siphoning, flux determinations began with the removal of a bath sample. Basal flux periods averaged about 2 hours. After the basal flux period had ended, serotonin (for a final concentration of 10^{-4} M/l) was added to the pondwater bath and carefully mixed such that the animal was not overly disturbed. After the bivalve had opened and was siphoning (within 15 minutes of serotonin addition), the serotonin flux was initiated by bath sample withdrawal. The average stimulated flux period was one hour then the final pondwater bath sample was removed. An average of 21% of ^{22}Na disappeared throughout the experimental period (during both the basal and 5-HT-stimulated sodium flux periods).

Sodium net flux was determined by monitoring the change in the sodium bath concentration ($c_o - c_x$, initial and final concentrations, respectively) of known pondwater volume (v) over a period of time (t). The bivalve soft tissue was removed, dried at 90°C until dry, and the dry tissue weight (w) ascertained. The net flux was expressed as μeq (g dry tissue · hr)$^{-1}$ and was calculated from the equation:

1. $$J_{net} = (c_0 - c_x) * (v) * (t)^{-1} * (w)^{-1}$$

Sodium influx was determined by monitoring the disappearance of ^{22}Na from the pondwater bath ($^{*}a_o$ and $^{*}a_x$, cpm at time zero and time x, respectively). The following equation was used to determine the influx:

2. $$J_{in} = (\ln (^{*}a_o / {}^{*}a_x)) * (\ln (c_o / c_x))^{-1} * J_{net}$$

The efflux was calculated by:

3. $$J_{out} = J_{in} - J_{net}$$

Steady state ($J_{net} = 0$) influx and efflux values ($J_{in} = J_{out}$) were calculated by the following equation:

4. $$J = (\ln (^{*}a_o / {}^{*}a_x)) * (v) * (c) * (t)^{-1} * (w)^{-1} \quad 4.$$

The adenylate cyclase (AC) activity assay is detailed in Scheide and Dietz (1983). Tissue was homogenized in 5 ml 50 mM/l tris-SO_4, pH=7.6 buffer by a Tissumizer (Tekmar) and centrifuged at 3000 x g for 20 min at 4°C. The crude homogenate pellet was washed in 2.0 ml tris-SO_4 buffer then resuspended in the tris-SO_4 buffer. A purified pellet was formed by an initial centrifuge step of the crude homogenate pellet at 100 x g for 20 min (4°C). The supernatant was decanted, then centrifuged and resuspended by the same methods used on the crude homogenate pellet. Adenylate cyclase activity was determined by incubating the resuspended pellet, after allowing the mixture to to warm to 25°C (10 min), in a mixture of 50 mM tris-SO_4, pH=7.6, 10 mM $MgSO_4$, 2 mM theophylline, 0.5 mM (ethylenediamine tetraacetic acid (EDTA), 0.5 mM adenosine 5' triphosphoric acid, 0.1 mM guanosine 5' triphosphoric acid, 2.5 mM creatine phosphate , 2.5 units per tube creatine phosphokinase and other additions noted in the results (neurotransmitters and $CaCl_2$).

The reaction was initiated with the addition of the resuspended pellet and terminated after 5 min by the addition of 0.8 ml boiling water and boiling the reaction tube for 3-5 min. Cyclic AMP formed during the assay was quantified by use of a cAMP binding protein isolated from human red cells (Scheide and Dietz, 1983). Protein values were determined by the Lowery method (Lowery et al., 1951). Adenylate cyclase activity was expressed as pmol cAMP (mg protein · 5 min)$^{-1}$.

The AC activity was determined using various species and tissues. Dilution of the crude homogenate pellet averaged 0.12, 0.16, 0.14, and 0.17 g original wet gill tissue weight per ml for Anodonta grandis, Carunculina texasensis, Corbicula fluminea and Ligumia subrostrata, respectively. Dilution of various Ligumia subrostrata tissue crude homogenate pellet averaged 0.18, 0.21, 0.13, 0.13, and 0.14 g original wet tissue weight per ml for gill, mantle, foot, heart, and muscle, respectively.

Calcium values were determined by centrifuging the resuspended homogenate pellet at 8,000 x g for 1 min and diluting a known volume of supernatant in 1% LaO_3-5% HCl. Calcium was ascertained on a Perkin-Elmer 303 atomic absorption spectrophotometer.

Cyclic AMP degrading enzymes were assayed by incubating approximately 300 pmol cAMP in a reaction tube with a final concentration of 50 mM/l tris-NO_3, pH=7.6, 10 mM/l $Ca(NO_3)_2$. Gill tissue from Ligumia subrostrata was homogenized in 3-5 ml 50 tris-NO_3, pH=7.6 buffer. Tissue fractions were the result of a 3000 x g centrifuge for 20 min (4°C). The pellet was resuspended in

tris-NO_3 buffer, 1 ml per 0.21 g wet tissue weight. The supernatant used was from a homogenate formed by 0.14 g wet tissue weight per ml. The enzymatic activity assay was initiated by the introduction of the homogenization fraction (final volume, 0.2 ml) and terminated by the addition of 0.8 ml boiling water followed with boiling the reaction tube for 3-5 min. The cAMP remaining was determined by use of the cAMP binding protein (Scheide and Dietz, 1983). The zero activity comparison was prepared by boiling the homogenization fraction (0.8 ml addition plus boiling water for 3-5 min), then adding tris-NO_3 / $Ca(NO_3)_2$ buffer and cAMP. Protein determinations were by the method of Lowery et al. (1951). Enzymatic activities were determined by the subtraction of cAMP quantified in the reaction tube from the zero activity tube and were expressed as pmol cAMP degraded (mg protein · 5 min)$^{-1}$. All assays were done in triplicate. Phosphodiesterase activity was determined by subtracting the cAMP present per reaction tube with 4.0 mM theophylline from the cAMP remaining in a non-theophylline containing reaction tube. The tube with theophylline represented nonspecific phosphatase (simply referred to as phosphatase) activity. Preliminary results indicated this reaction to be linear for the first 10 min. Phosphodiesterase activity was similar between 2 and 4 mM/l theophylline.

Statistical analyses utilized were the two-tailed student's 't' test and least-squares linear regression.

RESULTS

Several members of the family Unionidae and one of the Corbiculidae were observed to undergo a stimulation of sodium transport when serotonin (5-HT final concentration of 10^{-4}) was added to their pondwater bath (table 3). All four bivalve species exhibited the same "serotonin response" observed after the injection of 5-HT into the foot of the mussel (Dietz et al., 1982). The reaction to serotonin treatment includes an increase in siphoning, pronounced gape movements, an extension of the foot, and increased locomotory activity. The 5-HT stimulated sodium influx in each species was increased 2.5 to 5 fold over a basal sodium influx. In Carunculina texasensis, Corbicula fluminea and Ligumia subrostrata 5-HT stimulated the influx significantly above the efflux resulting in a significant ($P < 0.01$) net movement of sodium into the mussel. However, in Anodonta grandis, the sodium efflux was significantly increased ($P < 0.01$), in addition to the significant stimulation of the influx, contributing to a net loss of sodium. The net loss of sodium may indicate a serotonin effect on the excretory components of A. grandis, but may have been a consequence of fluid loss by the mussel's foot during gape and locomotory movements throughout the flux interval or the disturbance associated with sample withdrawal.

We have observed the forceful ejection of fluid from the ventral edge of the protruded foot when mechanically stimulated.

Table 3. Control and 5-HT (10^{-4} M/l) stimulated Na transport in 4 representative species of mussels in pondwater. The control flux of each individual was determined immediately prior to the addition of 5-HT.

		SODIUM FLUX $\mu eq\ (g\ dry\ tissue \cdot hr)^{-1}$					
		Control			5-HT Stimulated		
Species	n	J_{net}	J_{in}	J_{out}	J_{net}	J_{in}	J_{out}
Anodonta grandis	6	-0.27 ± 0.35^{A}	0.45 ± 0.29	0.72 ± 0.63	-1.63 ± 0.63	2.29 ± 0.66^{B}	3.92 ± 0.33^{C}
Carunculina texasensis	6	-0.41 ± 0.10	0.63 ± 0.20	1.04 ± 0.19	1.55 ± 0.32^{C}	2.01 ± 0.32^{CE}	0.46 ± 0.16^{B}
Corbicula fluminea	5	-1.89 ± 0.93	4.62 ± 0.85	6.51 ± 1.52	8.18 ± 0.94^{D}	10.59 ± 1.28^{CE}	2.41 ± 0.65^{B}
Ligumia subrostrata	5	0.67 ± 0.83	1.18 ± 0.65	0.51 ± 0.25	5.00 ± 0.50^{D}	5.60 ± 0.77^{CE}	0.60 ± 0.32^{B}

A Values represent the mean $\pm$ the standard error of the mean.

Values significantly different from basal,
B $P < 0.05$
C $P < 0.01$
D $P < 0.001$.

Values of J_{in} and J_{out} significantly different
E $P < 0.01$.

Analysis of the fluid indicated that blood-like fluid was being expelled (table 4). The expelled fluid represented approximately 64% and 47% of blood values in A. grandis and L. subrostrata, respectively. The low mean values of the expelled fluid and sample variability were the result of dilution by pondwater present in the mantle cavity and gill chamber, however when a fluid stream was directly collected the ionic concentration was higher than the fluid taken directly from the ventral edge of the foot (18.8 $\pm$ 0.1 meq/l for A. grandis, n=2). The Na, Cl and Ca concentrations were much higher than those in the pondwater (between 5 to 17 times greater) leading to the conclusion that blood was being expelled.

Adenylate cyclase (AC) activity was measured in the gill tissue homogenate pellet of all four species (figure 11). Serotonin specifically increased observed AC activity ($P < 0.01$), with a 3 to 5 fold increase from the basal activity being observed in A. grandis, C. fluminea and L. subrostrata. In C. texasensis, the increase in AC activity by 5-HT was greater than 30 fold above basal, but in each species, the 5-HT stimulation resulted in a similar activity. Basal AC activity levels were significantly lower ($P < 0.01$) in C. texasensis, when compared to basal values of A. grandis, C. fluminea and L. subrostrata. The trends in AC activity normalized to Lowery protein concentration were the essentially unchanged when AC activity was expressed per gram wet tissue. Anodonta grandis, C. fluminea and L. subrostrata were observed to have similar gill homogenate pellet AC activities, for A. grandis, 1158 $\pm$ 289 versus 3658 $\pm$ 925 (n=6), for C. fluminea, 1147 $\pm$ 319

Table 4. Blood and foot ventral edge fluid collected from Anodonta grandis and Ligumia subrostrata after a 1.5 hr exposure to 10^{-4} 5-HT pondwater. Pond water values for the ions were 0.7, 1.2 and 0.3 mM/1 for Na, Cl and Ca, respectively.

		ION CONCENTRATION mM/1						
		Blood				Foot Fluid		
Species	n	Na	Cl	Ca	n	Na	Cl	Ca
Anodonta grandis	10	19.3 ± 0.7[A]	17.0 ± 0.6	3.7 ± 0.2	4	12.2 ± 4.0	10.2 ± 3.6	2.6 ± 0.7
Ligumia subrostrata	11	20.1 ± 0.3	13.7 ± 0.4	3.5 ± 0.1	4	9.2 ± 1.9	6.2 ± 1.6	1.7 ± 0.3

A Values represent the mean ± the standard error of the mean.

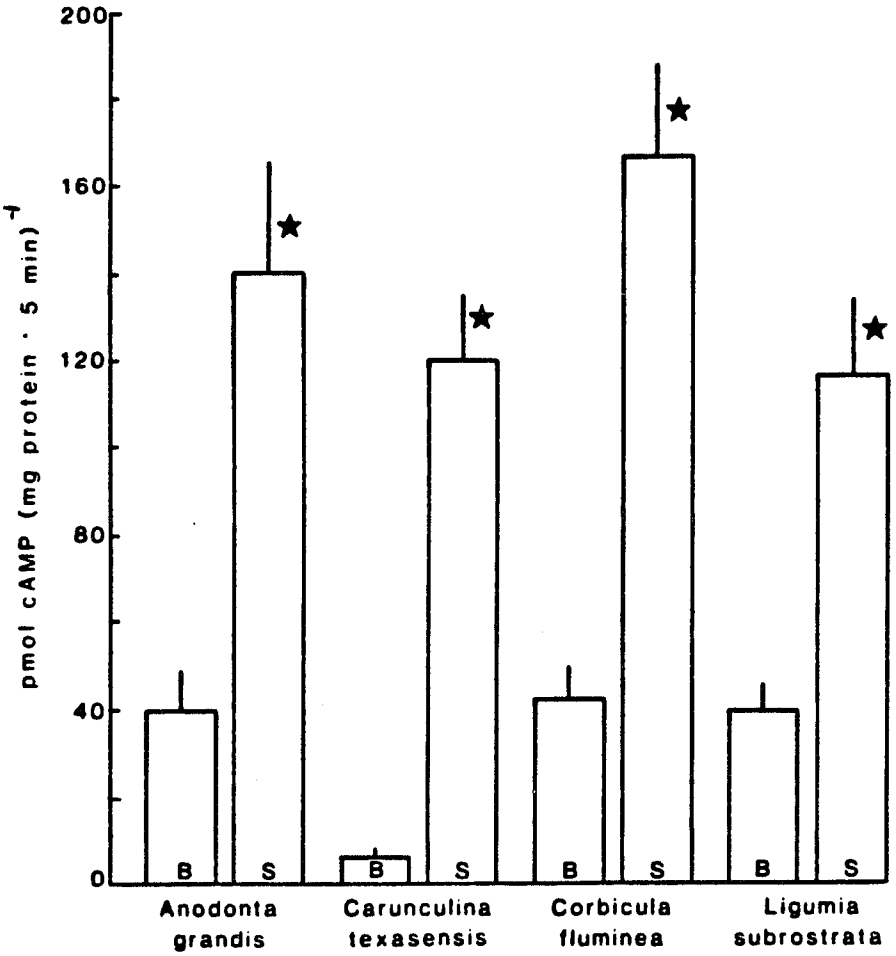


Figure 11. Basal (B) and 5-HT (S) (60 μM/l) stimulated AC activity in 4 species of freshwater mussels. Each corresponding bar represents the mean of 5 Anodonta grandis, 4 Carunculina texasensis, 4 Corbicula fluminea, and 6 Ligumia subrostrata, gill tissue homogenate pellet assayed in triplicate. The vertical lines represent the standard error of the mean. ★ = significantly different from corresponding basal values, $P < 0.01$.

versus 4384 ± 1048 (n=4), and for L. subrostrata, 1499 ± 202 versus 3566 ± 439 (n=6) (pmol cAMP (g wet tissue · 5 min)$^{-1}$, basal and 5-HT stimulated AC activities, respectively). Carunculina texasensis exhibited 75.9 ± 46.5 versus 2828 ± 391 pmol cAMP (g wet tissue · 5 min)$^{-1}$ for basal and 5-HT AC activities (n=4).

Adenylate cyclase was present in all L. subrostrata tissues studied (figure 12). Serotonin, is a major neurotransmitter substance in bivalves and it significantly stimulated AC activity in each tissue. Basal activities observed in the gill, mantle, heart, and posterior adductor muscle were similar as were their 5-HT stimulated activities displaying a 2.2 to 4.6 fold increase over basal AC activity. However, foot muscle basal and 5-HT stimulated AC activities were significantly higher ($P < 0.02$) than the other tissues. The same trends were evident when AC activities were expressed per gram wet tissue as were observed when normalized to Lowery protein.

Adenylate cyclase activity values have been observed to be quite variable from one gill tissue to another (this study, Scheide and Dietz, 1983). To better understand this variability, AC activity was compared to endogenous pellet calcium content (figure 13). Observed AC activity was inversely dependent on the endogenous calcium in the homogenate pellet. Basal ($r=0.77$, $P < 0.001$), serotonin ($r=0.88$, $P < 0.001$) and dopamine ($r=0.79$, $P < 0.001$) stimulated AC activities were inversely related to endogenous calcium with the calcium appearing to have a greater effect on the monoamine (5-HT and dopamine) stimulated activity than the basal

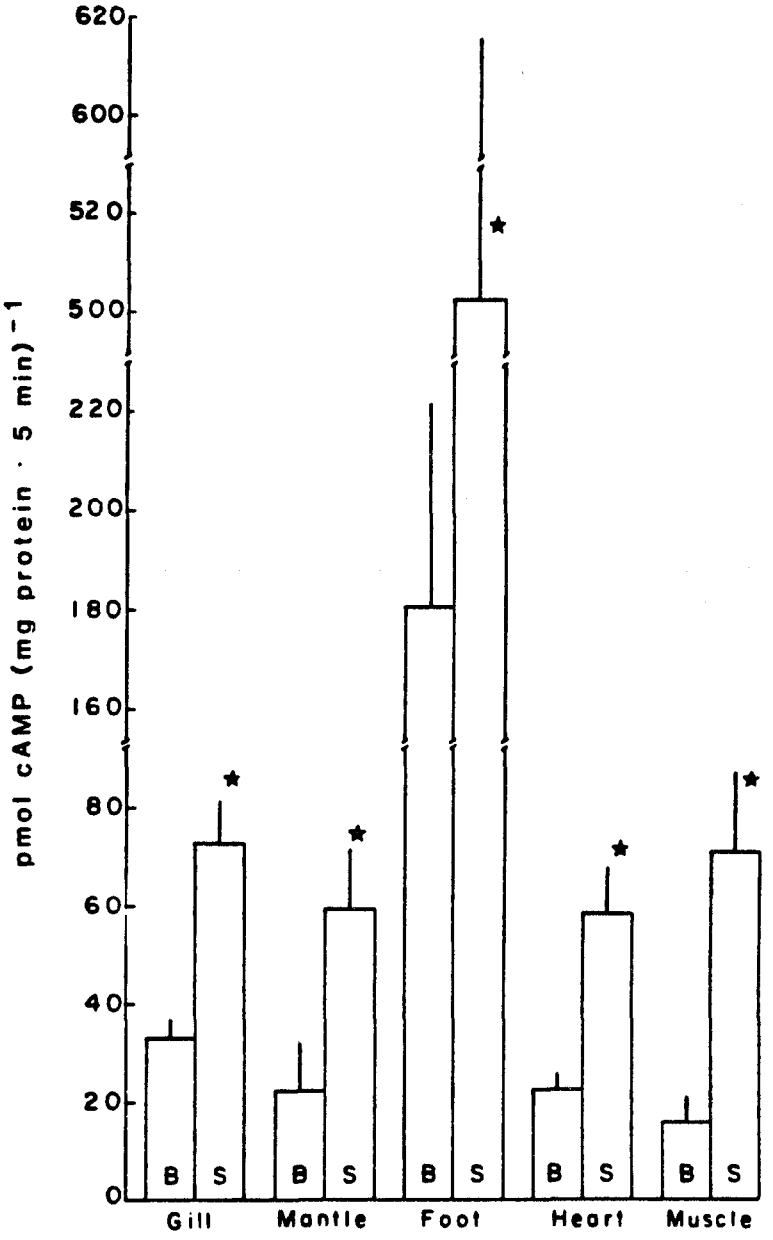


Figure 12. Basal and 5-HT (60 uM/l) stimulated AC activity in the various tissues of Ligumia subrostrata. The bars represent the mean of 7 gills and 3 each of mantle, foot, heart, and posterior adductor muscle, each assayed in duplicate. Vertical lines represent the mean standard error. ★ = significantly different from corresponding basal values, $P < 0.05$.

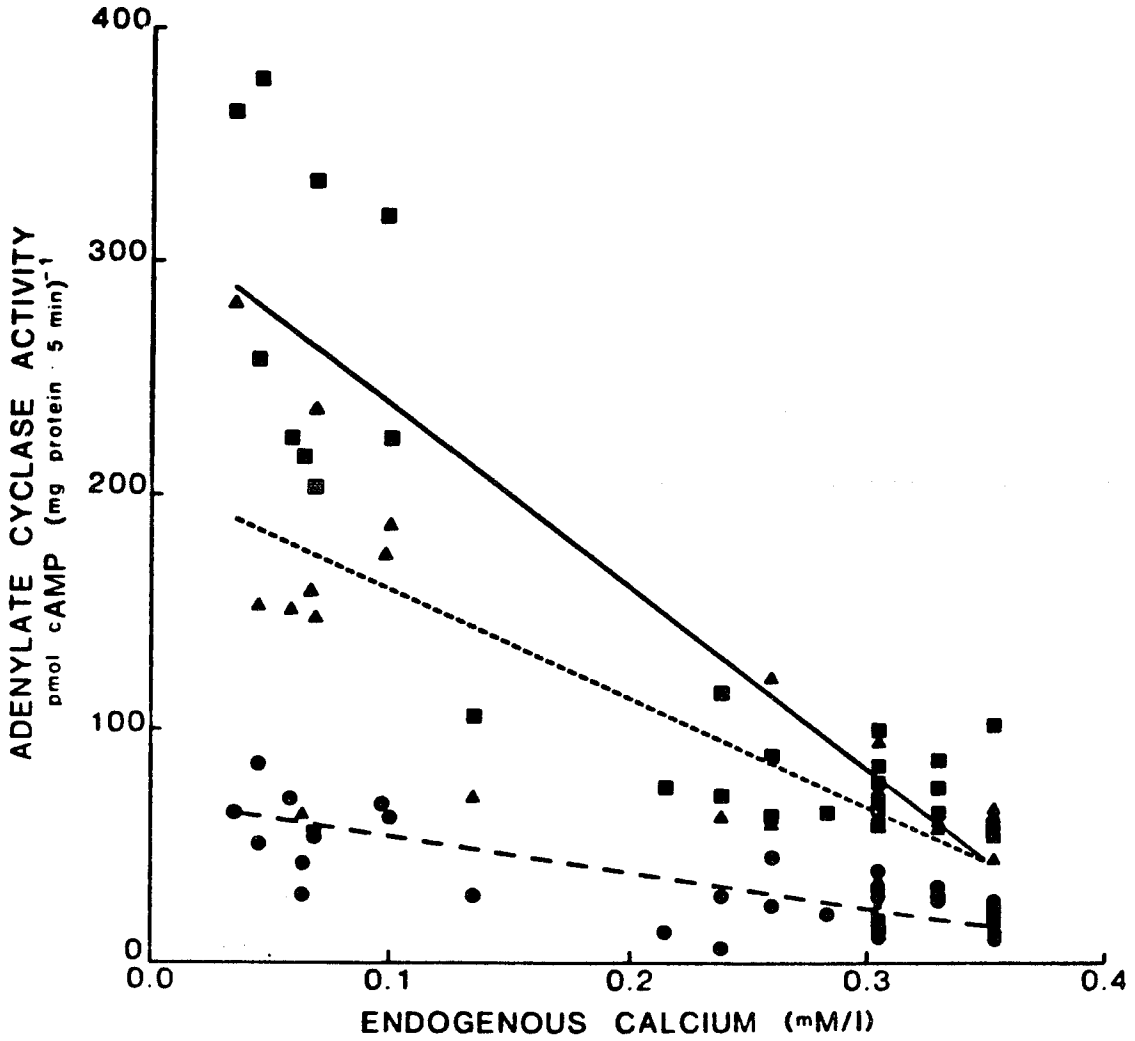


Figure 13. Gill adenylate cyclase activity varies inversely as a function of endogenous calcium in Ligumia subrostrata. Each point represents the mean value of 3 determinations. ●—● = basal ($y = -130 + 63(x)$, $r=0.77$, $P < 0.001$, $n=33$), ▲---▲ = 10 µM/l dopamine ($y = -463 + 205(x)$, $r=0.79$, $P < 0.001$, $n=24$), ■—■ = 10 µM/l serotonin ($y = -763 + 312(x)$, $r=0.88$, $P < 0.001$, $n=32$) AC activities with the lines fit by linear regressions.

activity. Basal AC activity linear regression was calculated to have a slope of -130 (pmol cAMP (mg protein · 5 min)$^{-1}$) / (mM Ca/l) while both 5-HT and dopamine stimulated activities were higher (-763 ± 75 and -463 ± 19 (pmol cAMP (mg protein · 5 min)$^{-1}$ / (mM Ca/l), respectively). The effect of calcium on the monoamine stimulated activities was significantly different ($P < 0.01$) from basal, with the most pronounced effect evident with 5-HT. Tissue calcium may be a very important modulator of gill tissue adenylate cyclase.

Crude homogenate pellet AC activity was increased by including a low spin centrifuge (100 x g for 20 min) step prior to centrifuging the homogenate at 5000 x g for 20 min. This procedure removes most of the large cells, cellular fragments and the large calcium concretions. The resuspended pellet AC activity of the purified pellet was significantly increased ($P < 0.001$) over the crude homogenate pellet (figure 14). Endogenous calcium levels were indicative of the AC activity observed for each group, the crude homogenate pellet averaged 0.26 mM Ca/l and the purified pellet averaged 0.07 mM Ca/l. The increased activity was evident in paired basal and monoamine stimulated AC observations, with the AC activities observed of the purified pellet being 2.5-3.4 fold greater.

Prostaglandin E_2 inhibits the sodium influx (Graves and Dietz, 1982; Saintsing and Dietz, 1983) and could possibly inhibit adenylate cyclase. The addition of PGE_2 did not effect basal purified pellet AC activity. In the presence of 50 ng/ml (140 μM/ml) PGE_2, AC activity was 68.4 ± 2.7 while basal was 57.5 ± 4.0

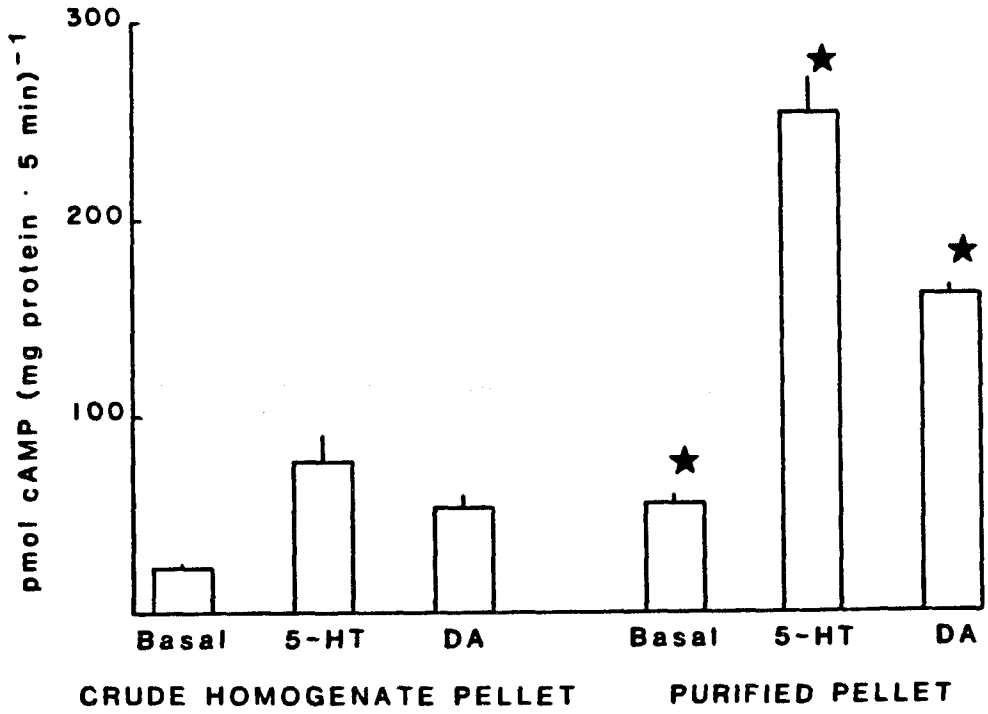


Figure 14. A comparison between the crude homogenate and purified pellet AC activities in Ligumia subrostrata. Bar values represent 3 gills, each assayed in triplicate, for basal, 10 μM/l 5-HT and 10 μM/l dopamine. The crude homogenate pellet and purified pellet were from the same gill tissue. Vertical lines represent the standard error of the mean. ★ significantly different from crude homogenate pellet, $P < 0.001$).

pmol cAMP (mg protein · 5 min)$^{-1}$ (n=2 gills each assayed in triplicate). Additionally, the inclusion of PGE_2 did not effect 5-HT or dopamine stimulated AC activity.

Exogenous calcium (in the form of $CaCl_2$) inhibited purified pellet AC (figure 15). The effect of exogenous calcium was primarily on the monoamine stimulated AC activities. Monoamine stimulated AC activities were significantly inhibited ($P < 0.01$) to approximately 50% normal. Normally, 5-HT and dopamine stimulated AC activity was 4.8 and 3.2 times above basal, but in the presence of $CaCl_2$, monoamine stimulated AC activity (above $CaCl_2$ basal) was only 3.5 and 2.0, respectively. Basal AC activity was slightly affected by the addition of $CaCl_2$. The addition of exogenous calcium (50% effective concentration 2.5 mM/l) was not as effective as the endogenous calcium (0.1-0.3 mM/l calcium). This may indicate the presence of an integral calcium regulatory component.

Phosphodiesterase and non-specific phosphatase (referred to as phosphatase) activities were investigated in the crude homogenate pellet and supernatant to determine whether these enzyme systems were relevant to the variability exhibited by adenylate cyclase in Ligumia subrostrata gills (figure 16). Phosphodiesterase and phosphatase activities were similar in both pellet and supernatant fractions, however they were significantly different ($P < 0.001$) between fractions. Supernatant phosphatase activity was increased over 4.5 x that of pellet while phosphodiesterase was increased 2.5 x. The summation of both enzyme activities was 267 $\pm$ 67 pmol cAMP degraded (mg protein · 5 min)$^{-1}$ (n=9) in the pellet fraction and a

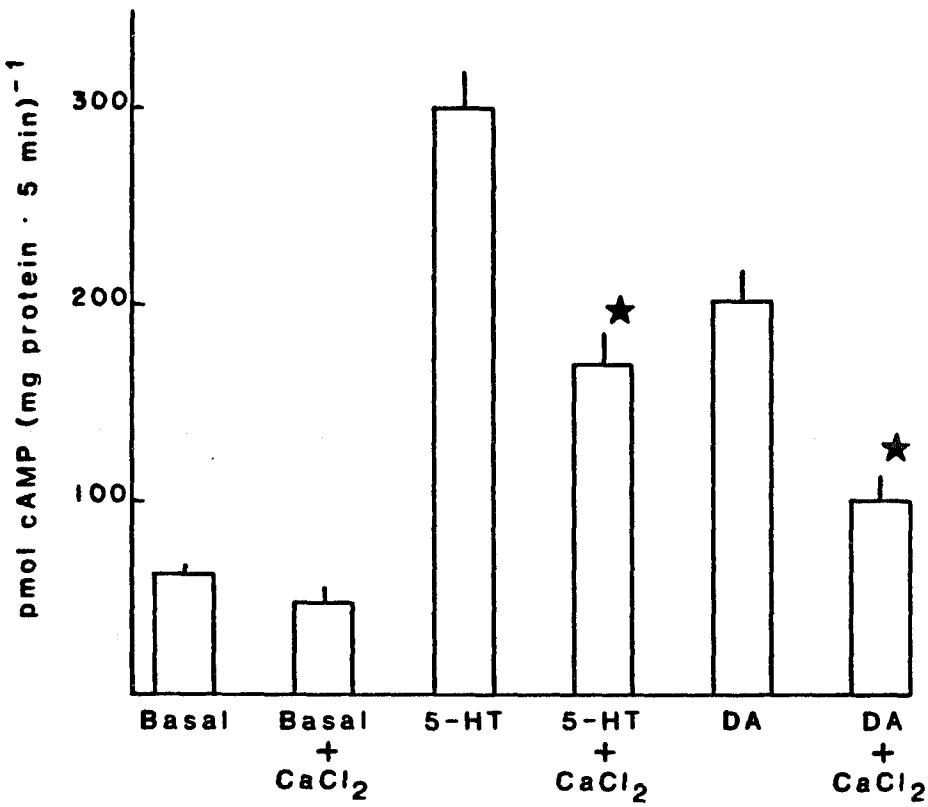


Figure 15. Ligumia subrostrata gill AC activity of basal, 10 μM/l 5-HT, and 10 μM/l dopamine with or without 2.5 mM/l $CaCl_2$. Each bar represents the mean of 4 gills, each assayed in triplicate. The vertical lines indicate the mean standard error. ★ significantly inhibited from the the normal condition, $P < 0.01$.

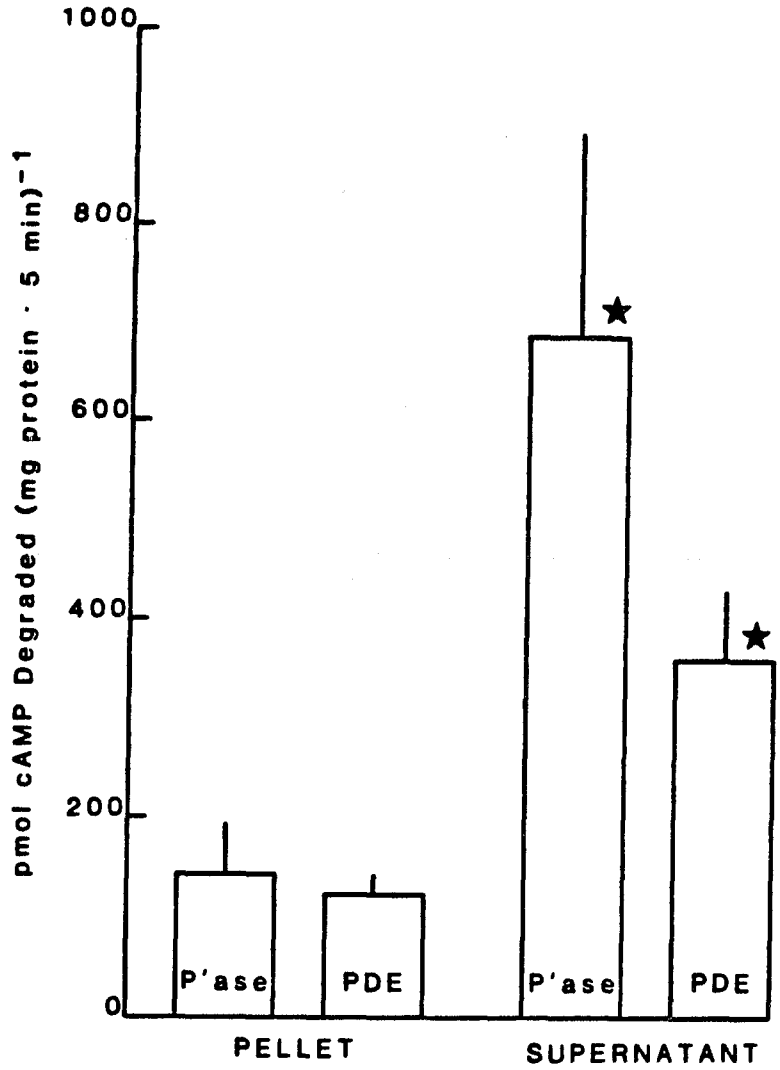


Figure 16. Nonspecific phosphatase and phosphodiesterase activities in the pellet and the supernatant fractions formed by a 3000 x g centrifuge of Ligumia subrostrata gill. Bar values represent the mean of 9 gill tissues for the pellet fraction and 3 gill tissues for the supernatant fraction, each assayed in triplicate. Vertical lines represent the mean standard error. ★ significantly different from the same enzyme system in the pellet, $P < 0.001$.

significantly higher ($P < 0.001$) 1038 $\pm$ 213 pmol cAMP degraded (mg protein · 5 min)$^{-1}$ in the supernatant fraction. Since the cAMP degradation enzymes were measured under ideal conditions, the pellet fraction does not appear to have highly active cAMP catabolic enzymes for cAMP concentrations present in the AC activity assay.

DISCUSSION

In freshwater mussels the sodium influx is stimulated by 5-HT and is correlated with the presence of a 5-HT-stimulated adenylate cyclase. Basal flux values presented are consistent with sodium transport values previously reported (Dietz, 1979), while the 5-HT stimulated values compare with those observed in salt depleted mussels (Murphy and Dietz, 1976; Dietz, 1978; McCorkle and Dietz, 1980; Scheide and Dietz, 1982). We have reported on the sodium influx increase in non-salt depleted L. subrostrata and C. texasensis, when injected with dibutyryl cAMP, 5-HT and dopamine (Graves and Dietz, 1982; Dietz et al., 1982). In addition, L. subrostrata gill tissue has both 5-HT and dopamine stimulated AC activity (Scheide and Dietz, 1983). However, only 5-HT stimulates the sodium influx in the isolated gill preparation (Dietz et al., 1982), even though both 5-HT and dopamine increase cAMP in the isolated gill (Chapter 3). Both serotonin and dopamine control gill ciliary motility (Paparo and Murphy, 1975, Paparo, personal communication), while serotonin also regulates sodium uptake by activating a cAMP enzyme system responsible for increasing the rate

of active sodium transport. Since 5-HT stimulates Na transport and also stimulates gill AC activity in two different bivalve families (Unionidae and Corbiculidae), the serotonin-adenylate cyclase coupled sodium transport may be present in most ion regulating bivalves.

Serotonin stimulated AC activity was present in all tissues investigated in Ligumia subrostrata. Since serotonin is a ubiquitous neurotransmitter substance in bivalves (Hiripi, 1968, 1972; Sweeney, 1968; Smith, 1982), 5-HT is responsible for many functions including: regulation of gill sodium transport (Dietz et al., 1982), control of ciliary motility (Gosselin, 1961; Paparo and Murphy, 1975), relaxing of the adductor muscles (Salanki and Hiripi, 1970), rhythmic activities (Hiripi and Salanki, 1973), and heart contraction (Higgins, 1971; Painter and Greenberg, 1982).

The comparatively high 5-HT stimulated AC activity in the foot muscle, when compared to the other tissues, may be due to adaptations for the control of burrowing and locomotion. Burrowing in bivalves is accomplished by the adductor muscles relaxing, resulting in the opening of the valves and the foot being protruded (Trueman, 1966; Trueman et al., 1966). The foot, during 5-HT stimulation, is quite active and the hemodynamic control exhibited requires complicated coordination (the shunting of blood in the blood sinus and muscle contractions). Gill tissue, while functioning to procure food, maintain salt balance, and carry on some gaseous exchange, is acting in a more uniform fashion. The AC activity difference then may be more a function of differential

activity control in the foot as opposed to other tissues.

Calcium concretions may contribute to AC activity variability by several means. Protein bound to the concretion matrix is structural, not functional, contributing to the overestimation of protein involved in enzymatic activity, reducing the observed AC values. Calcium bound to the concretions will become free in solution (Istin and Girard, 1970), thus inhibiting AC activities. Istin and Girard (1970) report 2 pools of calcium exist in mantle tissue, ionized and bound calcium. Exchange between these pools occurs and may be inhibited by Diamox, implying carbonic anhydrase mediation. Assuming a similar system occurs in the gill tissue as with mantle tissue and since carbonic anhydrase is present in gill tissue (Henry and Saintsing, 1983), free calcium levels will increase in the resuspended homogenate pellet with time.

Gill calcium regulates AC activity (basal, 5-HT- and dopamine-stimulated) in the in vitro preparation and may contribute in vivo. Calcium inhibition (0.1 to 0.5 mM/l) of AC activities has been observed in other AC systems (Marumo and Edelman, 1971; Hynie and Sharp, 1971; Bockaert et al., 1972; Brostrom et al., 1977; Litosch et al., 1982; Schmidt et al., 1982). Most reports involve the addition of calcium to the system, in this study endogenous calcium is causing the inhibitory effect. Endogenous calcium present in the crude homogenate pellet is attributed to the calcium concretions that contribute 25% of the dry tissue weight (Silverman et al., 1983). Gill AC levels are significantly reduced about 70% from 0.1 mM/l activity levels at an endogenous Ca level of 0.3 mM/l

and purified pellet AC activity is 50% inhibited by 2.5 mM/l exogenous Ca. The high level of calcium may be overestimating the effect of calcium on AC activity due to an increase in the formation of Ca-ATP and not Mg-ATP (Birnbaumer,1973). In each observation, the Ca effect was more pronounced on monoamine stimulated AC activity. Ligumia subrostrata gill AC activity is not only Ca sensitive, but the AC coupled-5-HT/dopamine receptors may be Ca-sensitive as has been observed in the control of Mytilus edulis lateral cilia (Paparo and Murphy, 1975).

Calcium is typically associated with the activation of phosphodiesterase activity. Theophylline (in the concentration used in determining AC activity) inhibited 50% of the gill cAMP catabolic activity. Similar inhibition of phosphodiesterase activity was observed in Mercenaria mercenaria ventricle (Hess et al., 1981). The remaining cAMP degrading activity was defined as phosphatase activity and should be constant in all similar preparations. In the gill homogenate pellet, the enzymatic degradation activities do not indicate the importance of this cAMP catabolic activity to the contribution of AC activity measurements. It is interesting to note the high level of cAMP degradative activities occurring in the homogenate supernatant, which agrees with the observation that the enzymes are found predominately in the soluble fraction of rat skeletal muscle (Gain and Appleman, 1978) or rat cerebral cortex (Kakiuchi et al., 1978). Supernatant AC activity, although greater than zero, was not significantly stimulated by 5-HT (Scheide and Dietz, 1983), perhaps as a consequence of the cAMP degradation

activities.

The purified pellet was used to determine the effect of PGE_2 on gill tissue AC. Prostaglandin E_2 inhibits sodium transport and PGE_2 synthesis may be inhibited by injections of serotonin or dibutyryl cAMP, thus reversing the PGE_2 effect (Saintsing et al., 1983). Crude homogenate pellet AC activity was unaffected by PGE_2 (Scheide and Dietz, 1983), however the sodium influx stimulatory system (5-HT) and the inhibitory system (PGE_2) appear to to be linked (Saintsing and Dietz, 1983). The purified pellet preparation was used to minimize the calcium interference. Application of exogenous PGE_2 to the purified pellet did not significantly change AC activity from normal values, an observation similar to that reported for the rat outer medulla (Jackson et al., 1980). It must be assumed that PGE_2 does not influence adenylate cyclase directly, but may indirectly influence AC enzymatic activity possibly by metabolism to an another form or altering cellular calcium concentrations. Calcium inhibits vasopressin stimulated AC activity in the toad bladder (Hynie and Sharp, 1971) and hamster kidney (Marumo and Edelman, 1971). Calcium also inhibits the vasopressin induced increase of toad bladder epithelial cAMP (Omachi et al., 1974). The rate of AC activity is altered by calmodulin (Ausiello and Hall, 1981). In addition, calcium channel blockers have been shown to directly influence the cAMP enzyme system at various levels (Levine et al., 1983). The effect of calcium on Na transport is unclear in freshwater bivalves, although preliminary data indicate a

Ca-Na exchange (Scheide and Dietz, unpublished observations).

Sodium transport is positively modulated by a serotonin-cAMP system in freshwater bivalves. The Na regulatory system functions to maintain sodium balance during periods of ion depletion, normal ion loss or water gain (the consequence of inhabiting a freshwater environment) or fluid loss. Regulation of Na in freshwater mussels is a rapidly acting mechanism (within 15 min) and may continue for several hours (Chapter 3). The serotonin mechanism must be an integral part of the circadian rhythms exhibited by freshwater bivalves. Ion transport (Graves and Dietz, 1980; McCorkle-Shirley, 1982) and oxygen consumption/activity rhythms (Hiripi and Salanki, 1973; McCorkle et al., 1979) all exhibit coincidental phase relationship, being elevated during the dark phase and depressed during the light phase. Coordination of these activities is probably ganglionic, but at this time, no area has been specifically identified as a sodium receptor for monitoring and regulating blood Na levels.

SUMMARY

1. Sodium influx is stimulated within 15 min when serotonin is added to the pondwater bath of 4 species of freshwater bivalves.
2. Each of the 4 species of bivalves has a 5-HT stimulated adenylate cyclase in the gill.
3. Serotonin stimulated AC activity is present in the 5

tissues observed in this study.

4. Endogenous calcium inhibits AC activity in a concentration dependent fashion in the crude homogenate pellet.
5. Exogenous calcium inhibits purified pellet AC activity.
6. Phosphodiesterase and nonspecific phosphatase activities are comparatively low in the homogenate pellet fraction and high in the supernatant fraction.

CHAPTER 3

SEROTONIN INFLUENCES ON SODIUM TRANSPORT AND OSMOTIC BALANCE

ABSTRACT

Unidirectional Na transport stimulation by 5-HT (0.1 mM/l) is similar to Na stimulation resulting from sodium depletion. Serotonin stimulated Na influx kinetics (K_m = 0.09 mM/l and J_{max} = 4.33 μeq (g dry tissue · hr)$^{-1}$) are similar to salt depleted mussels. Serotonin also changes water permeability, the mussels increasing in body weight by 0.36 ml H_2O (g dry tissue · hr)$^{-1}$ over controls. Amiloride completely inhibited the 5-HT stimulated Na influx, and the treatment of amiloride was reversible. Isolated gill tissue incubated in pondwater with 0.1 mM/l 5-HT reached maximum cAMP concentration by 1 min and declined in cAMP concentration over 20 min. The addition of 1.0 mM/l theophylline maintained the elevated gill cAMP concentration. Additions of 5-HT, dopamine, epinephrine or norepinephrine (0.1 mM/l in pondwater) increased gill cAMP concentrations, while L-dopa did not. Both 5-HT and dopamine significantly increased gill cAMP concentrations at 10^{-5} M/l.

INTRODUCTION

Animals that inhabit the freshwater environment must ion regulate to maintain ionic and osmotic homeostasis. Since freshwater is typically low in ionic concentrations, freshwater animals must have transporting epithelia capable of withdrawing ions from the environment into the animal, a requirement for a high ion transport affinity (Kirschner, 1972). Freshwater bivalves have a high affinity transport mechanism responsible for the uptake of sodium (Dietz, 1978). The site of Na uptake in freshwater mussels is the gill (Dietz and Findley, 1980; Dietz and Graves, 1981).

Recently it was observed that the injections of several monoamines stimulated the Na influx of pondwater acclimated mussels above control, an observation normally occurring after a mussel has undergone salt depletion (Dietz, et al., 1982). Incubation of isolated gill indicates that only serotonin (5-HT) was responsible for the increase in the Na influx (Dietz and Graves, 1981; Dietz et al., 1982). Injections of dibutyryl cAMP and theophylline also stimulated the Na influx (Graves and Dietz, 1982). Gill tissue has 5-HT stimulated adenylate cyclase and gill tissue cAMP concentrations were related to the sodium net flux (Scheide and Dietz, 1983), implying a 5-HT-cAMP mechanism mediating the stimulation of the Na influx.

In this paper, observations of the stimulation of the Na influx in pondwater acclimated freshwater mussels by 5-HT will be compared

to published observations of sodium depleted mussels (Murphy and Dietz, 1976; Dietz, 1978; Scheide and Dietz, 1982), under conditions of sodium influx kinetics and inhibition by amiloride, a diuretic that inhibits epithelial Na transport. The effects of monoamine on gill cAMP content will be investigated and compared to observations relating to the 5-HT stimulated adenylate cyclase responsible for the regulation of sodium transport.

MATERIALS AND METHODS

Mussels, Carunculina texasensis and Ligumia subrostrata, were transferred from ponds located near Baton Rouge to aerated aquaria containing a defined pondwater (0.5 mM/l NaCl, 0.4 mM/l $CaCl_2$, 0.2 mM/l $NaHCO_3$ and 0.5 mM/l KCl). Both species were used in this study, since the transport physiology of these mussels is similar (Dietz, 1978; 1979). Mussels were allowed at least one week to acclimate to laboratory conditions, light regime and temperature. Mussels were salt depleted by being in DW for several weeks (Murphy and Dietz, 1976; Scheide and Dietz, 1982).

Unidirectional sodium transport was determined by radiotracer analysis (Chapter 2). Mussels were rinsed for 1 to 2 hours in deionized water (DW) prior to any transport or water gain investigation. Sodium transport was determined by placing the mussel in a known volume of bath and sampling the bath over a period of time. After the last sample was collected, the mussel tissue was removed from the valves and dried at 90°C for at least 18 hr. The

net Na flux was determined by the difference in the bath Na (measured by flame photometry) over time and normalized to the mussel's dry tissue weight (ueq (g dry tissue · hr)$^{-1}$. The Na influx was determined by monitoring the disappearance of ^{22}Na from the bath. The efflux was calculated by subtracting the Na net flux from the Na influx. Serotonin (5-HT) was added to pondwater to a concentration of 10^{-4} M/l. Amiloride and serotonin were dissolved in 1.0 mM/l NaCl and the pH was adjusted to 7.0 with the addition of tris-SO_4 (approximately 0.3 mM/l).

Determination of water gain was accomplished by weighing the mussels at the onset of the experimental period and 4 hr later. Each mussel was removed from the DW rinse, the valves carefully pried open and the mussel gently shaken to remove any mantle or gill water. Water on the shell was blotted dry and the mussel was weighed to within 0.01 g. At the termination of the weight gain period the weighing process was repeated. Fecal material was not visually present in the bathing medium, thus any weight difference was assumed to be the result of H_2O loss or gain. Paired analysis (Student's 't') was used to compare the initial and final weight differences.

Gill cAMP concentrations were determined from male Ligumia subrostrata. Mussels were selected for large gill size. Tissue preparation and incubation methods were similar to those used in the isolated gill flux (Dietz and Graves, 1982). The mussels were opened by excising the adductor muscles, gill pairs were rapidly removed and placed in pondwater. The anterior ends were trimmed to

insure only gill tissue was being studied. Each gill pair was separated and transversely bisected such that each mussel provided 8 pieces of gill tissue. Before incubation in 10 ml of pondwater plus monoamine, gill tissue was blotted and weighed. Incubation times ranged from 1 to 20 min and experimental temperature was 25 $\pm$ 1°C. Termination of the incubation was accomplished by removing the gill tissue from the incubation vial, placing the tissue in 1 ml 0.8 N perchloric acid, 40% ethanol and 1 mM/l EDTA and immediately homogenizing the tissue with a tissumizer (Tekmar) (Ellington, 1981). The resulting homogenate was centrifuged at 10,000 x g for 10 min (4°C). The supernatant was withdrawn and and the pellet was digested in 1.0 N NaOH for protein determination (Lowery et al., 1951). Phenol red (10 µl) was added to the supernatant and it was neutralized to a pH of 6.5 to 7.0 by the addition of 3 M K_2CO_3, 50 mM MES. The resulting precipitate was separated by centrifuging at 10,000 x g for 20 min (4°C). The cAMP content of the supernatant was quantified by use of a cAMP binding protein assay (Scheide and Dietz, 1983). Gill tissue pieces were used in a random order and a set of mussel gill tissue from one animal took 20 min to process. There were no observable statistical differences between gill tissue pieces from the 4 gills of a mussel.

Statistical analyses used were the paired Student's and two sided Student's t test. Least-squared linear regression analysis was utilized to determine the Lineweaver-Burke values.

RESULTS

Serotonin (5-HT) has been demonstrated to stimulate the unidirectional Na influx in freshwater mussels (Chapter 2). Sodium influx kinetics of Carunculina texasensis stimulated by 5-HT (0.1 mM/l) were characterized by placing mussels in bathing media having various concentrations of NaCl (figure 17). Stimulated sodium transport was saturable and Michealis-Menton kinetic constants were calculated from a Lineweaver-Burke plot. An affinity (k_m) of 0.09 mM/l and a maximal sodium influx (V_{max}) of 4.73 μeq (g dry tissue · hr)$^{-1}$ were calculated from the linear regression line formed by the Lineweaver-Burke values ($r=0.87$, $P < 0.001$, $n=31$). Significant stimulation ($P < 0.001$) of the Na influx ($J_i > J_o$) occurs at 0.1 mM/l NaCl (2.66 $\pm$ 0.2 versus 0.94 $\pm$ 0.14, for J_i and J_o, respectively, $n=3$). At maximal stimulation, J_i/J_o exceeded 7.

In addition to stimulating the sodium influx, 5-HT was observed to change water permeability (table 5). Mussels maintained in 5-HT (10^{-4} M/l) pondwater for 4 hours significantly increased in weight over control mussels. In a paired comparison, both groups of mussels were different in weight from initial values with the control group losing weight significantly ($P < 0.001$) and the 5-HT group significantly gaining weight ($P < 0.001$). A total difference of 0.36 ml H_2O (g dry tissue · hr)$^{-1}$ was calculated between control and 5-HT stimulated mussels.

Amiloride was observed to effectively inhibit the sodium influx

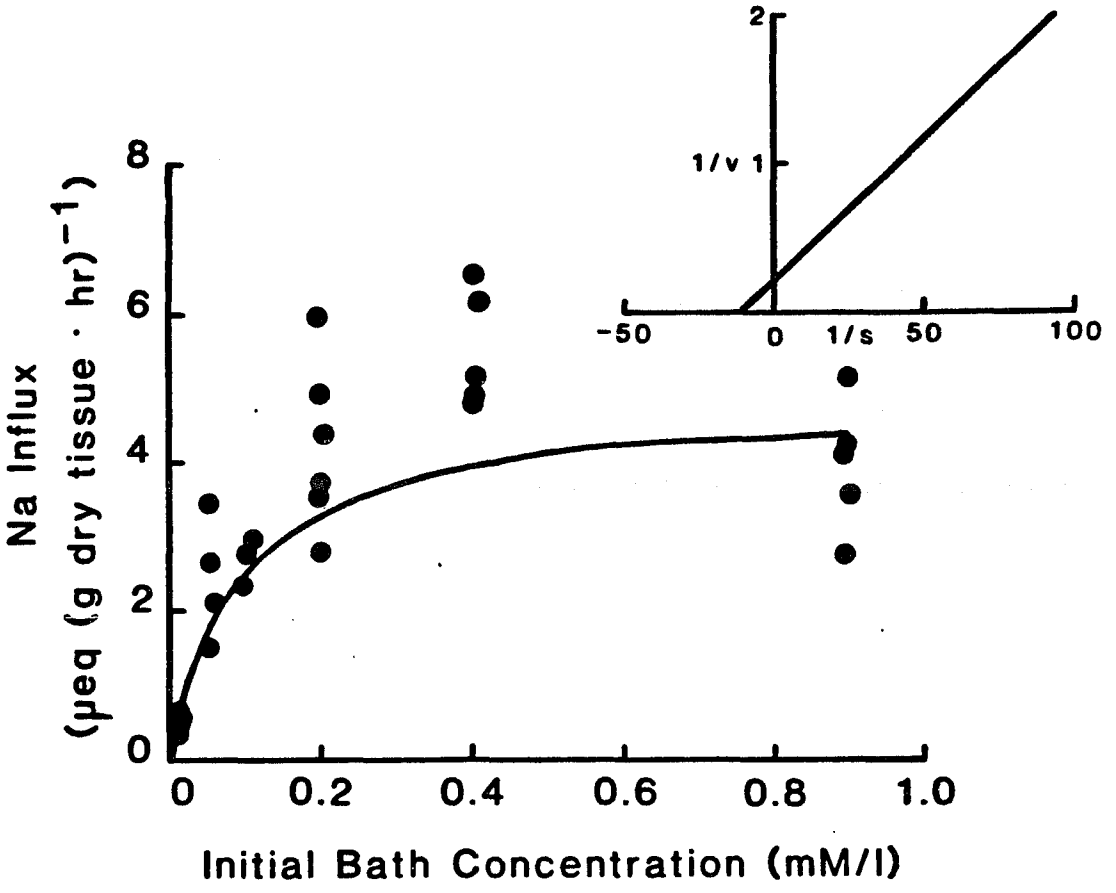


Figure 17. Kinetics curve for the 5-HT stimulated (10^{-4} M/1) Na influx. The line represents the best fit curve of the linear regression (r=0.87, P < 0.001) of the Lineweaver-Burke plot in the insert.

Table 5. Mussel weight change over a 4 hour period in pondwater with or without 10^{-4} serotonin.

	n	ml H_2O (g dry tissue)$^{-1}$ BEGIN	FINAL	DIFFERENCE
Pondwater Control	14	11.70 ± 0.80[A]	11.27 ± 0.81[B]	-0.43 ± 0.07
Pondwater Serotonin	13	13.10 ± 1.52	14.09 ± 1.49[B]	0.99 ± 0.15[C]

A Values represent the mean ± the standard error of the mean.

B Significantly different ($P < 0.001$) from beginning weight.

C Significantly different from control ($P < 0.001$).

stimulated by 5-HT (10^{-4} in an 1.0 mM/l NaCl bath) or dibutyryl cAMP (injected dose 1.0 μM (g dry tissue)$^{-1}$) (table 6). Sodium influx in the stimulated mussels (5-HT or dibutyryl cAMP) was inhibited 90% while the sodium efflux was uneffected. The Na influx of 5-HT and dibutyryl cAMP treated mussels were significantly greater than the Na efflux (J_i/J_o being 3.5 and 3 for 5-HT and dibutyryl cAMP). In the 5-HT and dibutyryl cAMP with amiloride treatments the influx to efflux ratio was 0.45 and 0.44. The amiloride effect was reversible. When mussels injected with dibutyryl cAMP then placed in 1.0 mM/l NaCl with 5 X 10^{-4} amiloride (Na influx = 0.58 $\pm$ 0.05 μeq (g dry tissue · hr)$^{-1}$) were returned to 1.0 mM/l NaCl without amiloride, Na influx values immediately returned to the stimulated values (5.95 $\pm$ 1.66 μeq (g dry tissue · hr)$^{-1}$, n=4).

Isolated gill tissue cAMP concentrations declined when they were incubated in pondwater for 20 min (figure 18). The decrease in gill cAMP levels was more pronounced with 5-HT stimulation. Initially (within 1 min), the gill cAMP concentration was increased 2.5 x basal when the gill tissue was incubated in the presence of 10^{-4} M/l 5-HT in pondwater, however after 20 minutes the 5-HT elevation above basal was 1.4 x. The addition of 1 mM/l theophylline to the incubation medium was effective in maintaining elevated gill cAMP levels for 20 min in the isolated gill (table 7). Control gill cAMP concentrations were not significantly different.

Gill tissue cAMP concentrations were significantly elevated above basal when the gill tissue was incubated in pondwater containing 5-HT, dopamine, epinephrine and norepinephrine (figure

Table 6. Unidirectional sodium flux of mussels in 1.0 mM/l NaCl. Mussels were stimulated by 5-HT (10^{-4} M/l) in the medium. Dibutyryl cAMP was injected into the foot of the mussel (1.0 μmol (g dry tissue)$^{-1}$). Amiloride (5 X 10^{-4} M/l) was dissolved into the bath.

	n	μeq (g dry tissue · hr)$^{-1}$		
		J_{net}	J_{in}	J_{out}
Control	11	-0.52 ± 0.26[A]	1.18 ± 0.39	1.76 ± 0.30
5-HT	12	3.66 ± 0.76[B]	5.13 ± 0.80[BC]	1.47 ± 0.35
Amiloride + 5-HT	12	-0.60 ± 0.19	0.50 ± 0.08	1.10 ± 0.21
Dibutyryl cAMP	8	3.90 ± 0.50[B]	5.81 ± 0.82[BC]	1.91 ± 0.79
Amiloride + Dibutyryl cAMP	4	-0.76 ± 0.49	0.58 ± 0.05	1.33 ± 0.50

A Values represent the mean ± the standard error of the mean.

B Significantly different from control and corresponding amiloride treated mussels, $P < 0.01$.

C Significantly different from J_{out}, $P < 0.01$.

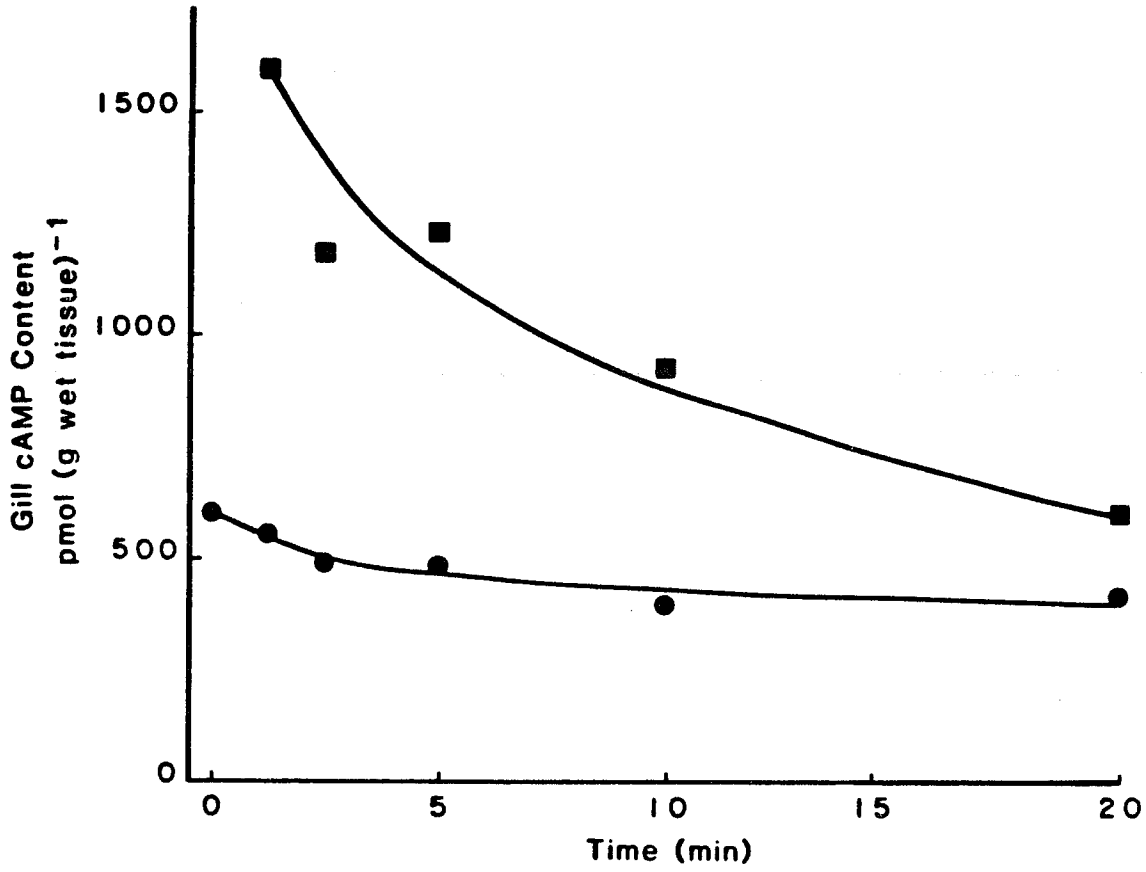


Figure 18. Changes in gill cAMP concentrations for control (●) and 5-HT treated (■) isolated gill tissue as a function of time. All points represent the mean of 2 gill values except time 0 (n=8) and 5 min (n=4).

Table 7. Basal and 5-HT stimulated gill cAMP concentrations after a 20 min incubation with or without 1.0 mM/l theophylline.

Treatment	pmol (g wet tissue)$^{-1}$	pmol (mg protein)$^{-1}$
Control	438.2 ± 56.9[A]	9.09 ± 1.39
Theophylline + Control	560.6 ± 67.5	11.06 ± 1.87
5-HT	815.0 ± 79.9[B]	15.31 ± 1.69[B]
Theophylline + 5-HT	1308.7 ± 191.3[BC]	26.52 ± 4.27[BC]

A The mean of 6 mussel gills ± the standard error of the mean.

B Significantly different from cooresponding control, $P < 0.02$.

C Significantly different from 5-HT treatment, $P < 0.05$.

19). Incubation in L-dopa did not change the gill cAMP concentration from basal. Typically, 5-HT and dopamine incubations elevated gill cAMP levels greater than epinephrine or norepinephrine. The same relationships were observed when gill cAMP values were normalized to mg protein on g wet tissue. Values for one min incubation in pondwater containing monoamine were 10.8 $\pm$ 0.6, 22.2 $\pm$ 7.1, 22.1 $\pm$ 5.5, 11.0 $\pm$ 1.7, 17.0 $\pm$ 2.1 and 16.6 $\pm$ 1.9 pM (mg protein)$^{-1}$ for basal, 5-HT, dopamine, L-dopa, epinephrine and norepinephrine, respectively. The gill cAMP concentrations observed in control and 5-HT or dopamine treated gills were significantly lower after a 20 min incubation, however the cAMP levels were still above basal ($P < 0.05$). Cyclic AMP concentrations in isolated gill tissue were stimulated in a dose-dependent fashion by 5-HT and dopamine (figure 20). Pondwater concentrations of 10^{-5} M/l of 5-HT and dopamine significantly increased ($P < 0.001$) gill cAMP concentrations.

DISCUSSION

The kinetic characteristics of unidirectional Na transport stimulated by 5-HT in freshwater mussels is similar to the transport occurring in salt depleted mussels based on maximum velocity of influx values and affinity. Stimulated Na influx values in Na depleted mussels (mussels maintained in sodium free media for longer than 2 weeks) range between 2.5 and 4.5 μeq (g dry tissue $\cdot$ hr)$^{-1}$

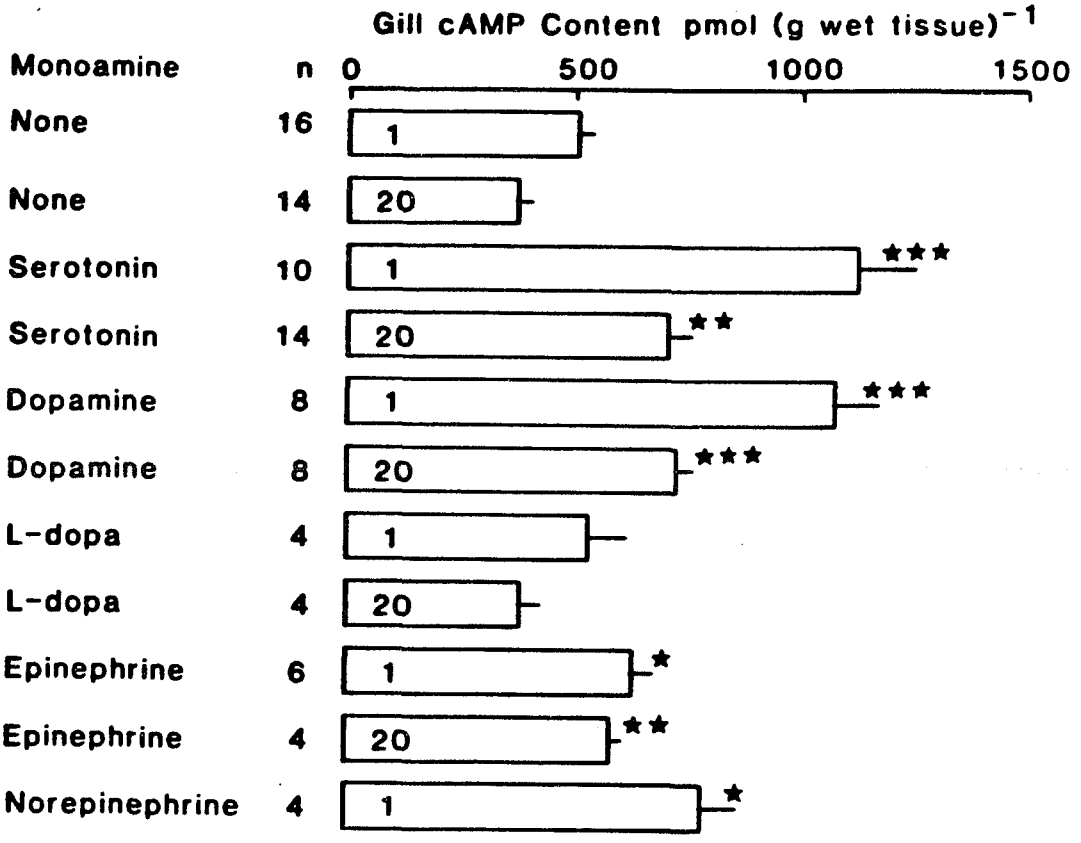


Figure 19. The effect of monoamine treatment and time on isolated gill tissue cAMP concentrations. Incubation time is indicated in each bar (1 = 1 min, 20 = 20 min). The horizontal bars represent the mean and the lines are the standard error of the mean. Significantly different from corresponding control, ★ $P < 0.05$, ★★ $P < 0.01$ and ★★★ $P < 0.001$.

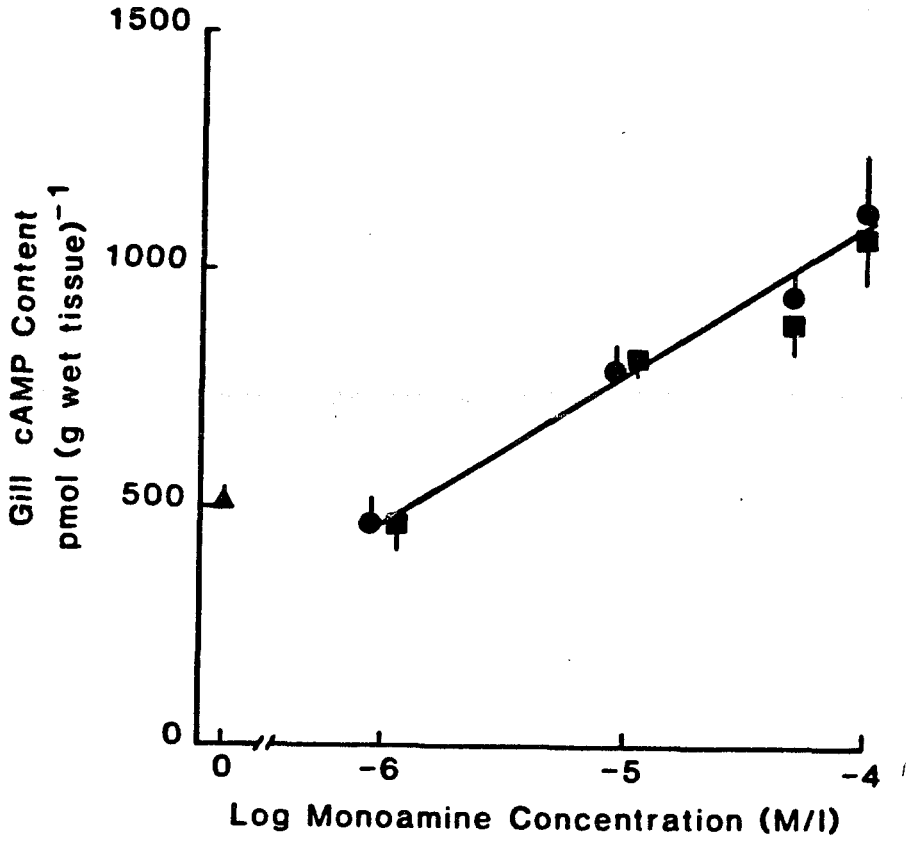


Figure 20. Effects of monoamine concentration on isolated gill tissue cAMP content (● = 5-HT or ■ = dopamine). The vertical lines represent the standard error of the mean.

(Dietz and Branton, 1975; Murphy and Dietz, 1976; Scheide and Dietz, 1982; 1983). Serotonin and dibutyryl cAMP treatments increased the Na influx 3.7 to 7.1 ueq (g dry tissue · hr)$^{-1}$ depending on the dose administered in mussels acclimated to pondwater (Dietz et al., 1982; Graves and Dietz, 1982; Scheide and Dietz, 1982; 1983). The range of Na influx values in 5-HT stimulated mussels is higher than sodium depleted possibly because 5-HT is maximally stimulating all active transport sites.

An affinity constant of 0.09 mM/l for the 5-HT stimulated influx is similar to the calculated affinity values for whole mussels, 0.15 mM/l in NaCl (unstimulated, Dietz, 1978) or the isolated gill preparation 0.17 and 0. 19 mM/l, for control and 5-HT stimulated gills (Dietz and Graves, 1981). The affinity observed here is within the range of K_m values reported of freshwater animals (Kirschner, 1972). Comparison of J_{max} values between 5-HT stimulated and sodium depleted mussels shows that the two are similar, but the J_{max} of 5-HT stimulated animals will be a function of the 5-HT dose administered. In control mussels (Table 2) a J_{max} calculated on wet weight basis is 111 nmol (g wet tissue · hr)$^{-1}$ and is similar to those observed in other representative freshwater animals (Kirschner, 1983) while the 5-HT stimulated value of 484 nmol (g wet tissue · hr)$^{-1}$ is higher (calculations based on personal observation that Ligumia subrostrata has 9.6 ml H_2O (g dry tissue)$^{-1}$). Transport stimulation is the result of an increase in the active transport mechanism not Na-Na exchange since there is a significant increase in the Na influx over the efflux. There is

probably an increase in the number of active transport sites, J_{max} since the affinity is not different from previously published values (Dietz, 1978).

Amiloride inhibition of sodium transport has been noted in many epithelial tissues (Kirschner et al., 1973; Kirschner, 1983). The addition of amiloride inhibits NH_4^+-H^+/Na exchange in these transporting epithelia, thus depressing the J_{max} (Maetz et al., 1976; Kirschner, 1979). Sodium influx in freshwater bivalves is coupled to an NH_4^+-H^+/Na exchange and the normal Na influx is inhibited by amiloride (Dietz, 1978). The inhibition observed in the 5-HT and dibutyryl cAMP stimulated mussels is similar to the control condition reported for Carunculina texasensis (Dietz, 1978). All of the stimulated transport is inhibited by amiloride treatment and the effect of amiloride was reversible.

Water permeability appears to change with the addition of 5-HT. Mussels gain water similar to the water permeability change observed in the vasopressin stimulated toad bladder (Orloff and Handler, 1962; Handler et al., 1965). Serotonin treatment of gill tissue increases cAMP, which may be an integral part of water permeability regulation. Salt depleted mussels exhibit fluid loss, the extracellular and the intracellular space H_2O is reduced (Murphy and Dietz, 1976). Thus, the movements of solute (Na) in or out of the salt depleted mussels was associated with readjustments in water balance.

Gill cAMP levels are increased with monoamine treatment. This observation was expected with 5-HT and dopamine, since these

monoamines stimulate gill adenylate cyclase (Scheide and Dietz, 1983), however epinephrine and norepinephrine elevated gill cAMP levels and have no effect on Ligumia subrostrata gill adenylate cyclase. All of these monoamines are present in freshwater bivalves (Hiripi, 1968; 1972) and stimulate whole animal Na influx (Dietz et al., 1982), however only 5-HT stimulates Na influx in the isolated gill preparation. Evidence presented here indicates that the monoamines are entering the gill and are physiologically active, however 5-HT is acting specifically to stimulate transport dependent adenylate cyclase. No stimulation of gill cAMP was observed with L-dopa and L-dopa has no effect on whole animal transport (Dietz et al., 1982) or adenylate cyclase (Scheide and Dietz, 1983). These neurotransmitters may be influencing the cAMP enzyme system by stimulating some of the 5-HT/dopamine neurons in the gill tissue or by pharmacological action on one of the other cAMP system enzyme (for example, phosphodiesterase).

Serotonin elevation of cAMP in gill tissue of Ligumia subrostrata has been associated with Na regulation (Dietz and Graves, 1981)and ciliary activity (Paparo and Murphy, 1975). Dopamine also regulates ciliary activity (Paparo and Murphy, 1975). The 5-HT/dopamine cAMP stimulatory system is very similar to that in Aplysia californica. Both 5-HT and dopamine increase cAMP levels in gill tissue (Kebabian et al, 1979) and stimulate adenylate cyclase (Weiss and Drummond, 1981). Dopamine modulates gill muscular contraction (Swaan et al., 1982) and in the freshwater bivalve, dopamine may also be functioning to control muscular activity in the

gill.

The gill cAMP elevation induced by 5-HT peaks by the first min of incubation. By inhibiting phosphodiesterase, theophylline treatment maintained the elevated cAMP concentrations in the gill. Injections of theophylline are also effective in stimulating the Na influx (Graves and Dietz, 1982). Cyclic AMP levels decreased in gills not exposed to theophylline during the 20 min interval, thus it important that a short flux period be used in order to correlate gill cAMP levels to Na influx.

Active sodium transport in mussel gills is mediated by neural rather than hormonal stimulation. Blood concentrations of 5-HT are comparatively low (about 5×10^{-8} M/l) compared to gill tissue content (about 5.8×10^{-6} M/l, Dietz, et al., 1982). Gill tissue has 5-HT in sufficient quantity to stimulate adenylate cyclase and yet maintain the blood concentration of 5-HT below the threshold concentration for stimulation of adenylate cyclase (Scheide and Dietz, 1983). The gill tissue of Ligumia subrostrata is innervated with 5-HT containing neurons (Dietz et al, 1983). Sodium transport stimulation by exogenous 5-HT is immediate in freshwater bivalves (Chapter 2). A sodium transport rhythm has been observed in freshwater mussels (Graves and Dietz, 1980; McCorkle-Shirley, 1982), indicating control by some coordinating mechanism probably ganglionic. In the "normal" mussel, the Na flux is regulated in a more controlled manner than exhibited when stimulated with exogenous 5-HT. Neural control permits a graded response for fine control of sodium homeostasis.

SUMMARY

1. Serotonin stimulated Na influx kinetics are similar to salt depleted mussels.
2. Water permeability is altered by 5-HT, with the mussels gaining 0.36 ml H_2O (g dry tissue · hr)$^{-1}$ over controls.
3. Amiloride inhibited the 5-HT stimulated Na influx.
4. Isolated gill cAMP content peaks by the first min of addition of 5-HT, then declines for the next 20 min and the decline may be inhibited by the addition of theophylline.
5. Additions of 5-HT, dopamine, norepinephrine or epinephrine increased gill cAMP content. Serotonin and dopamine were effective at 10^{-5} M/1.

APPENDIX

CYCLIC AMP BINDING PROTEIN

I. CYCLIC AMP BINDING PROTEIN SEPARATION

******All of this assay must be kept on ice or at 4°C. Cold temperature is critical for a good binding protein activity.******

1. Mix reagents one day prior to separation (see end of this document for the recipes). Refrigerate all reagents!
2. Cut the blood bag plastic tube and equally aliquot blood cells into 16 50 ml low speed centrifuge tubes.
3. Add saline buffer to each tube, filling them to 40 ml.
4. Centrifuge this mixture in the Beckman J-6B (1,000 x g, 10 min, 4°C).
5. Aspirate the supernatant. In aspirating the supernatant be sure to clean off any lipid material floating at the top of the supernatant. It is also imperative that the white cell layer be removed at this time (proteases abound in this layer). Add more saline buffer for a total volume of 40 ml per tube.
6. Repeat 2-5 until the supernatant is clear. Change centrifuge tubes every 2 runs to get rid of coagulated cells.
7. After 3 to 4 red cell washes, transfer the red cells to 50 ml high speed centrifuge tubes and lyse the red cells in phosphate buffer, pH 7.8 (PB7.8), again filling the tubes to a 40 ml total.
8. Weigh the tubes to match their weights. Centrifuge the tubes in a Sorvall RC-5B at 30,000 x g (no brake) for at least 15 minutes (4°C).

9. Aspirate the supernatant, add more phosphate buffer to total 40 ml.
10. Repeat steps 8 and 9 until a very white and fluffy "pellet" is obtained. Change centrifuge tubes every other run to eliminate the 'tight' pellet that contains proteases and phosphatases. After removing the supernatant, transfer the fluffy layer to a fresh centrifuge tube. Add 1-2 ml PB7.8 to the old tube. Swirl this volume carefully to resuspend any residual fluffy layer with care being taken not to disturb the tight pellet.
11. Once the proper pellet is obtained, mix the pellet with 1 to 20 ml PB7.5 and combine all pellets together.
12. Mix the pellet mixture 3:1 (NH_4Cl:pellet) with 1.0 M NH_4Cl and incubate this mixture for at least 2 hr at 4^oC.
13. Centrifuge (RC-5B, 30,000 x g, 15 min, 4^o) the ammonium precipitate and aspirate the supernatant.
14. Mix the pellet with 2 ml of the phosphate buffer (pH=7.5), swirl, pipette the mixture out, and pool the red cell membranes in a chilled 50 ml graduated cylinder, leaving the tight pellet in the centrifuge tube.
15. Add in a 1:1 ratio, a 1:1 solution of glycerol:phosphate buffer (pH=7.5) with .04 mg/ml RIA grade BSA (G:P Buffer)
16. Run a binding protein dilution curve (see below).
17. Aliquot the binding protein into small quantities based on the results of 16. Freeze and store the binding protein at -60^oC. If the preparation is fairly clean of proteases and the like contaminants, the binding protein may be stored for over 1 year.

18. Run a standard curve (see below) to ascertain the sensitivity of this particular cAMP binding protein.

RECIPES FOR NECESSARY SOLUTIONS

SALINE BUFFER

NaCl	0.15 M	21.02 g
KH_2PO_4	5 mM	0.27 g
K_2HPO_4	5 mM	1.74 g

Add NaCl to 2.4 l glass distilled water (GDW). Add the KH_2PO_4 to 400 ml and K_2HPO_4 to the rest of the NaCl solution. Add the 5 mM KH_2PO_4 to the 2 l until pH=7.8.

PHOSPHATE BUFFER, pH=7.8 (PB7.8)

KH_2PO_4	5.0 mM	0.41 g
K_2HPO_4	5.0 mM	3.48 g

Add KH_2PO_4 to 600 ml GDW and K_2HPO_4 to 4 l GDW. Add the 5 mM KH_2PO_4 to the 4 l until pH=7.8 pour out 3 l of this soution. To the approximately remaining one liter add the KH_2PO_4 to a pH=7.5 (PB7.5).

AMMONIUM CHLORIDE

NH_4Cl	1.0 M	10.7 g

Add NH_4Cl to 200 ml GDW.

GLYCEROL:PB7.5 BUFFER (G:P BUFFER)

Glycerol		100 ml
PB7.5		100 ml
RIA BSA	(4 g/ml)	2 ml

Add the above quantities together and mix very well. Store at -20°C.

II. CYCLIC AMP BINDING PROTEIN ASSAY

1. Number cAMP binding protein (cAMP BP) assay tubes and place then on ice.
2. Stepwise, dilute ^{3}H-cAMP from basic stock (20-30 Ci/mM ^{3}H-cAMP (the higher the activity the better the assay), Amersham, TRK 304) to 1 ml deionized water (label stock A).
3. Dilute with DW stock A to 13-15000 cpm per 50 µl sample.
4. Mix together in the following ratio: cAMP assay buffer:DW: ^{3}H-cAMP solution at 5:2:1 (0.25:0.1:0.05 ml per tube).
5. Aliquot 400 µl of the above mixture into each assay tube.
6. Place DW, standards or unknown 50 µl aliquots in the proper assay tubes.
7. To the zero tubes, add 50 µl of the BP dilution mixture.
8. Thaw, then dilute, the binding protein with the BP dilution mixture. The proper dilution was determined as a part of the BP separation procedure (see below) and is noted on the lid of the container as well as the date separated. Mix well and allow

several minutes for the bubbles to disappear.

9. Rinse the tip of the pipette several times prior to the first aliquot. Add 50 µl of BP to each assay tube (except the zero tube). Final volume is 500 µl.

10. Cap and vortex each tube. Allow 16 to 30 hours for equilibrium at 4°C.

11. To terminate the cAMP BP assay, add 100 µl of a well stirred 1% Mallinckrodt charcoal, 0.1% Dextran T-70 suspension. Vortex the tubes for 5 to 8 seconds each. Allow 4 to 4.5 minutes of charcoal exposure for each tube.

12. Centrifuge each tube at 8,000 x g for one minute. Remove the tubes and place them on ice.

13. Withdraw 500 µl from each tube and count.

14. The above procedure (#11-13) is performed in the cold room at 4°C.

15. Suggestion: Do 24 assay tubes at each time. Set both timer at 4.5 minutes. Activate timer 1. Add charcoal to 12 tubes, cap, vortex and place the tubes in the centrifuge. Wait till one minute remains on timer 1, then start timer 2. Add charcoal to to tubes 13-24. Cap and vortex the second group. When timer 1 sounds, start the centrifuge (usually after 2-4 tubes are vortexed). When the centrifuge stops, replace the group 1 assay tubes with group 2. Withdraw 500 µl aliquots form the first group. Start the centrifuge when the second timer sounds (usually between #4-6 aliquots). The first group should be aliquoted just as the centrifuge stops.

RECIPES FOR NECESSARY SOLUTIONS

BINDING PROTEIN ASSAY BUFFER (BPAB):

Tris	150.0 mM	18.17 g
$MgCl_2$ 6:H_2O	15.0 mM	3.05 g
EDTA	1.5 mM	0.29 g

Mix the above compounds to 970 ml DW, pH to 7.6 with concentrated HCl, add DW to 1 liter.

III. CYCLIC AMP BINDING PROTEIN DILUTION CURVE

***** Objective:To observe the total binding of ^{3}H-cAMP with a decreasing concentration of the cAMP binding protein. *****

1. Dilute the stock binding protein (BP) solution 2:1, 1:1, 1:2, 1:3, 1:4, 1:9, and 1:49 with G:P Buffer (stock BP:(G:P Buffer)).
2. Number 1.5 ml vials 1-18 and add 0.05 ul water to each one.
3. Add 13-15,000 cpm to each tube with buffer and DW (II.-4).
4. Add the corresponding binding protein dilution to the proper vial (BP:(G:P buffer)):

   1,2 no BP 3,4 stock BP 5,6 2:1 7,8 1:1 9,10 1:2

   11,12 1:3 13,14 1:4 15,16 1:9 17,18 1:49
5. Incubate overnight and harvest all 18 tubes at one time.

6. Plot the ^{3}H-cAMP binding curve (figure 21) and dilute the stock with G:P Buffer prior to daily use to the concentration that first causes the break in the curve plateau. Figure 21 demonstrates the curves of cAMP binding protein separated from several different pints of blood.

IV. CYCLIC AMP CONCENTRATION CURVE

Check the sensitivity of the newly purified binding protein to find out the proper range. Bracket between 0.01 to 10 pM. Use the methods outlined in the Cyclic AMP Binding Protein Assay. Figure 22 demonstrates the concentration curve of several assays. The specificity of the cAMP binding protein is demonstrated in figure 23. The ATP levels used in the Adenylate Cyclase Assay do not interfere with the cAMP Binding Protein Assay (any interference would be negated by the subtraction of zero).

V. ADENYLATE CYCLASE ASSAY

1. Rapidly excise gill tissue, blot, weigh and record the wet weight. Place the tissue in 5 ml homogenization buffer (HB). Maintain on ice.
2. Homogenize the gill tissue with two 10 second bursts on the Tissumizer. Centrifuge at 5,000 rpm with the SS-34 head on the Sorvall RC-5B for 20 minutes.

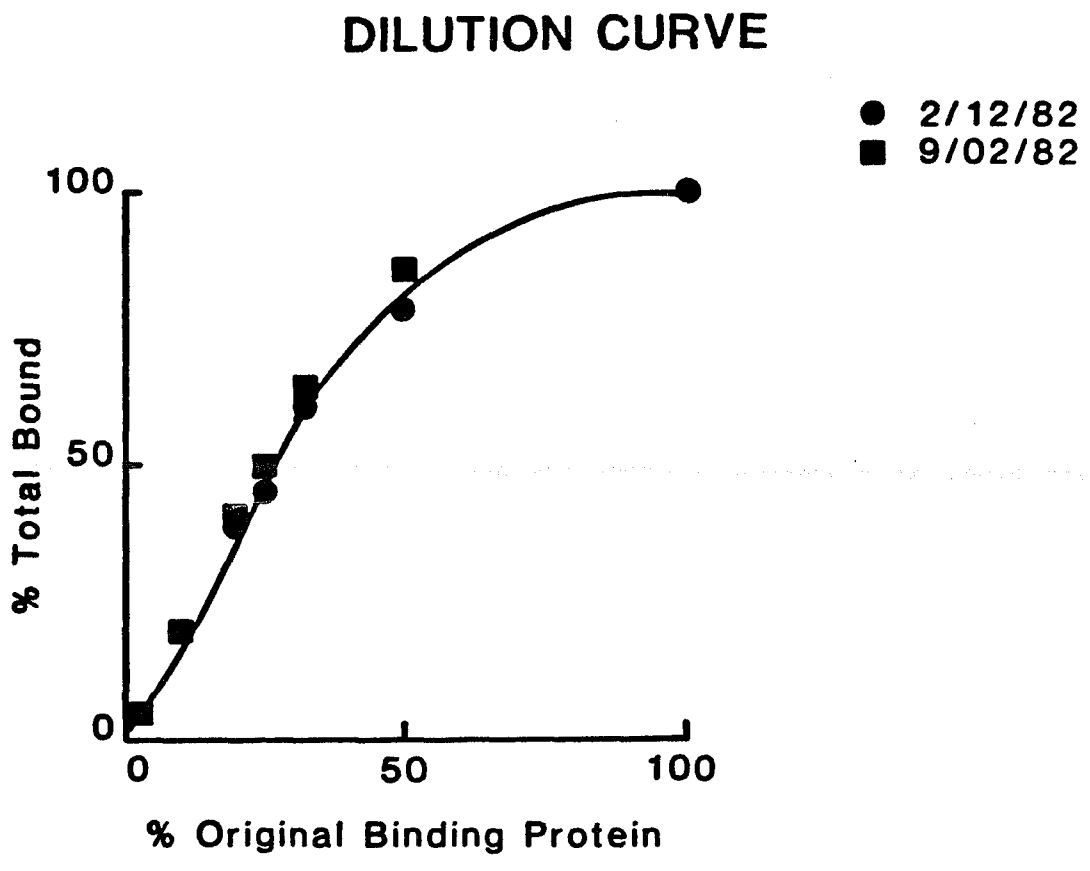


Figure 21. Binding curve of the separated cAMP binding protein from two different pints of blood. The binding activity is expressed as a percentage total bound.

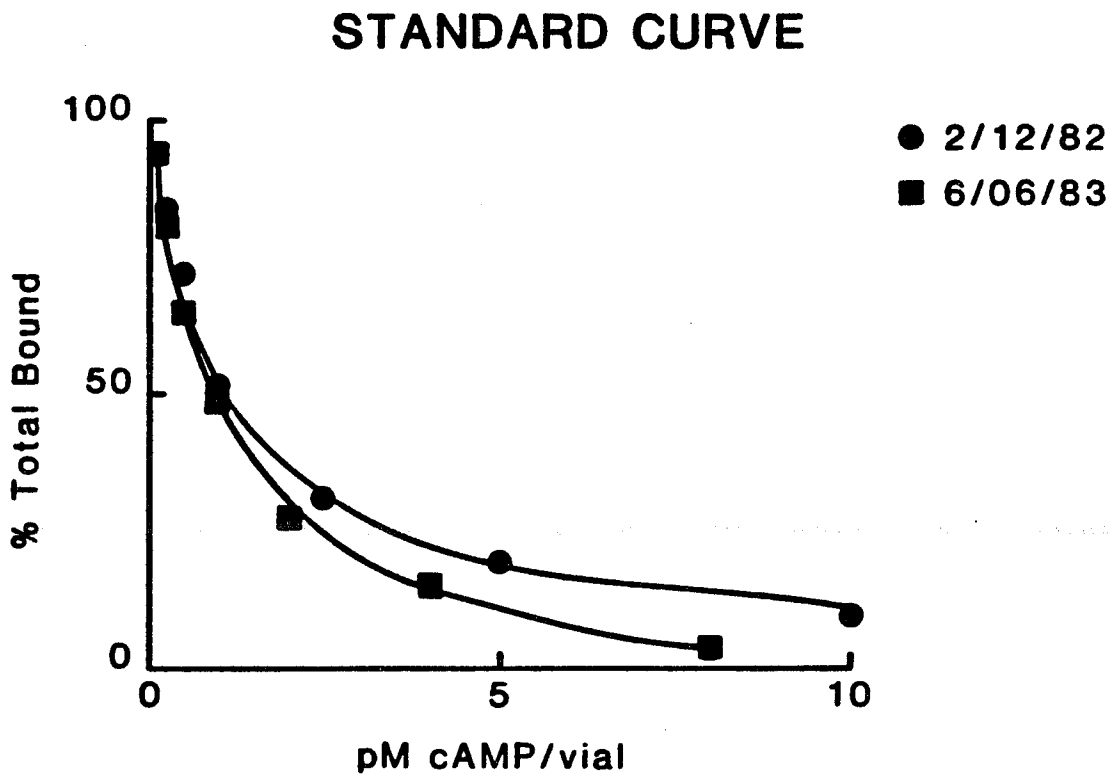


Figure 22. Dilution curve of cAMP representing the percentage ^{3}H-cAMP bound with unlabeled cAMP.

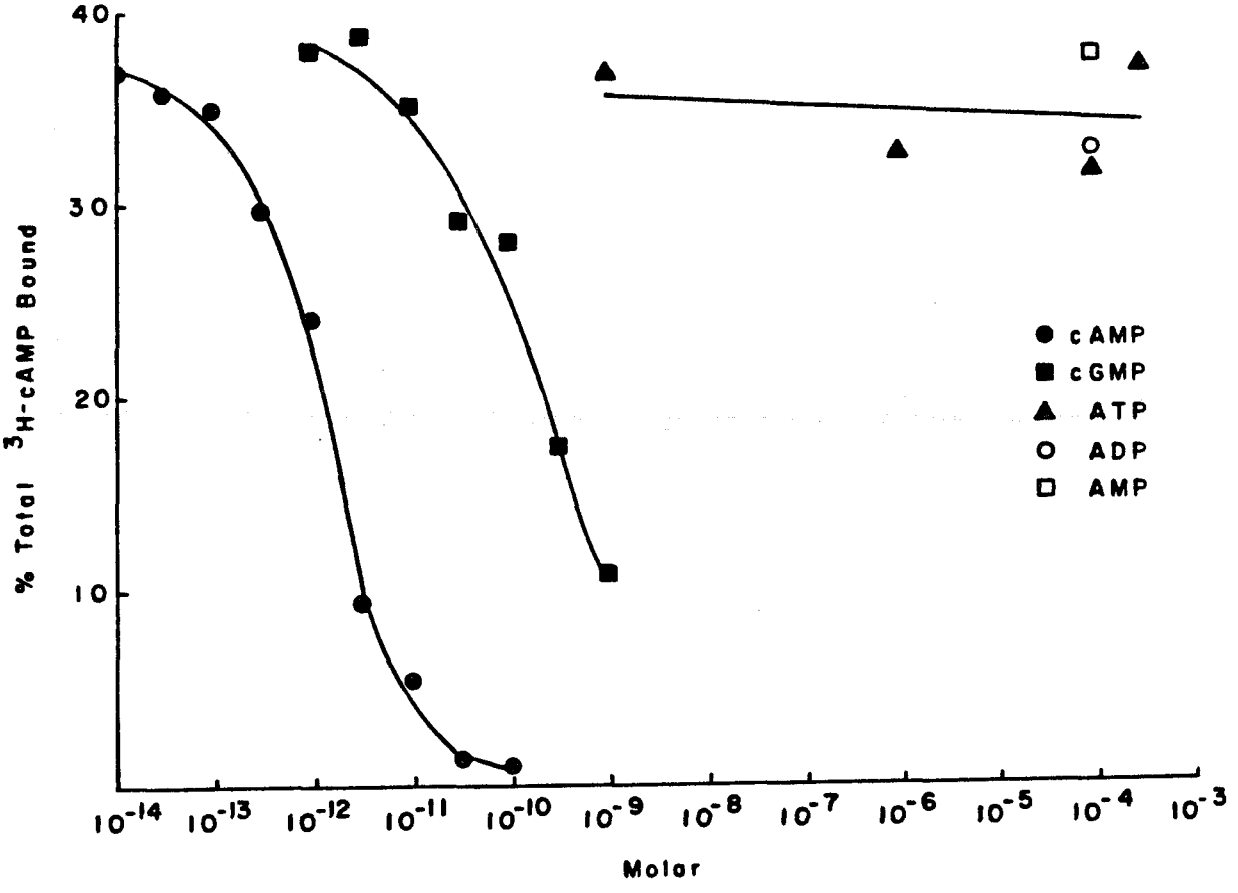


Figure 23. Specificity of the cAMP binding protein with various nucleotides.

3. Number and weigh the reaction tubes. The weight will be used for a dilution factor when the reaction is terminated.
4. Thaw out frozen adenylate cyclase assay buffer (ACAB) and creatine phosphokinase (CPK).
5. On a hot plate (set at 7 - 8) place a 250 ml and 50 ml beaker each filled with glass distilled water. These 2 must boil and the volume should never be less than 50%.
6. Mix additives, prepare them for a 4 fold dilution.
7. Pour out the supernatant of the centrifuged homogenate and add 2 ml HB. Swirl the 2 ml around and discard the liquid. For Ligumia subrostrata gill tissue, add 1 ml HB per 0.2 g original wet tissue. This ratio will vary between tissues and species.
8. Resuspend the pellet and transfer into a test tube. Allow 10 minutes for the suspension to warm up. Note: my observations were at $25^{o}C + 2^{o}C$, if the ambient temperature was not in this range a water bath was used. Measure room temperature were the reaction tubes are.
9. Add 100 µl CPK to 4 ml ACAB. Vortex gently, but thoroughly.
10. Add 100 µl ACAB + CPK mixture to each reaction tube except the zero tubes.
11. Add 50 µl of the additives to each tube (DW, neurotransmitter, etc.).
12. Just prior to the onset of the adenylate cyclase assay, add 50 µl aliquots to the zero tubes, add 0.8 ml boiling water and place the tube in boiling water.
13. Noting the time each addition occurs, add 50 µl of homogenate

pellet suspension to the reaction tube. This initiates the adenylate cyclase activity determination. Note: add the pellet suspension every 10 to 15 seconds, gently vortexing the pellet suspension each time before adding, and vortexing the reaction tube just after the addition.

14. To terminate the reaction add approximately 0.8 ml of boiling water from the 50 ml beaker (wash the boiling water up and down the pipette tip several times between each addition) and place the tube into the 250 ml boiling water bath.
15. Add 100 ul of ACAB + PCK to the zero tubes and continue cooking.
16. After 4 to 5 minutes, remove the reaction tubes and place on ice.
17. Weigh the reaction tubes and assume the weight difference as the dilution factor.
18. Centrifuge the tubes in a Beckman J-6B for 20 minutes at 3000 x g ($4^{o}C$).
19. Assay the supernatant for cAMP and digest the the pellet in 1.0 N NaOH for Lowery protein determination.

RECIPES FOR NECESSARY SOLUTIONS

HOMOGENIZATION BUFFER (HB):

Tris	50 mM	6.06 g

Add tris to 970 ml DW and titrate to pH=7.6 with either H_2SO_4 or HCl depending on which activity is being studied, ACAB should be

titrated with the same acid.

ADENYLATE CYCLASE ASSAY BUFFER (ACAB):

Tris	100.0 mM
$MgSO_4$	20.0 mM
EDTA	1.0 mM
ATP	1.0 mM
GTP	0.2 mM
Phosphocreatine	5.0 mM
Theophylline	4.0 mM

Mix all of the above except ATP, GTP and phosphocreatine to 40 ml DW. Titrate to 7.8 with H_2SO_4. Add the final reagents and DW to 50 ml, adjust the pH to 7.6.

CREATINE PHOSPHOKINASE (CPK):

1,000 Units diluted in 1 ml GDW.

ADDITIONS:

Mix and dilute in DW (4 fold dilution)

VI. TISSUE cAMP CONCENTRATION

1. Cyclic AMP extraction may be accomplished by a variety of methods. All involve the following:
   1. Homogenizing the tissue.
   2. Centrifuging the the homogenate at 10,000 x g (20 min, 4^{o}).

3. Assaying the supernatant for cAMP content by use of the cAMP binding protein method.

4. Use the pellet for dry weight or digest in 1.0 N NaOH for Lowery protein determination. The following are two methods used in this dissertation.

2. Tris buffer extraction.

1. Homogenize the tissue in an osmotically similar tris-SO_4, 1.0 mM/l EDTA (pH=7.6) buffer (not a Cl buffer).

2. Place the homogenate in a boiling water bath for at least 10 min. to destroy enzyme activity.

3. Use methods outlined in VI 1.2 to 1.4.

3. PCA extraction (Ellington, 1981).

1. Homogenize the tissue in a PCA mixture (outlined below).

2. Centrifuge at 10,000 x g (20 min, 4^{o}).

3. Decant the supernatant and add 10 µl phenol red to the supernatant (save the pellet for dry weight or protein determination).

4. Neutralize the supernatant with a K_2CO_3 mixture (recipe below) to a pH of 6 to 7 (bright yellow), normally 1 ml PCA mixture will require 200 to 250 µl of the K_2CO_3 mixture to be neutralized. Care should be taken not to go basic (purple to bright purple).

5. Remove the precipitate by centrifuging at 10,000 x g (20 min, 4^{o}C).

6. Withdraw the supernatant with a transfer pipette and weigh. Assume the weight to be the final volume.

7. Assay the supernatant. Neutralize 1 ml of of the PCA mixture and add the volume being quantified to all the nonsample tubes to maintain the same volume and cAMP binding protein environment.

4. Run a dilution curve of the supernatant by either method to determine which volume of the supernatant gives the best results (to avoid inhibition or competition by another compound).

RECIPES FOR NECESSARY SOLUTIONS

TRIS-SO_4 BUFFER

Tris	50 mM	6.06 g
EDTA	1 mM	0.29 g

Add tris and EDTA to 970 ml water. Adjust the pH to 7.6 with concentrated H_2SO_4, add water to 1 liter.

PERCHLORIC ACID MIXTURE

PCA (60%)	0.8 N	133 ml
Ethanol	40%	400 ml
EDTA	1.0 mM	0.29 g

Add the ethanol to 400 ml water, add EDTA, then add PCA, bring the volume to 1 liter with water.

K_2CO_3 MIXTURE

K_2CO_3	3.0 M	207.3 g
MES	50 mM	4.88 g

Add the K_2CO_3 and MES to a total volume of 500 ml.

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VITA

John Iver Scheide was born in Tacoma, Washington on January 25, 1953. He attended school in Washington and graduated from Mount Rainier High School in June, 1971. Undergraduate studies were begun in September, 1971 at Highline Community College for two years, then he enrolled at the University of Washington from which he graduated in June, 1976 with a Bachelor of Science in Zoology. He was admitted as a full time graduate student in Physiology at Louisiana State University. He attended Duke University Marine Laboratory for the course "Membrane Physiology and Osmoregulation". John graduated with a Master of Science in Physiology in August, 1980, then continued for a Doctor of Philosophy in Physiology. Both degree programs at Louisiana State University were directed by Dr. Thomas H. Dietz. During his tenure at LSU, John taught laboratory courses in General Biology, General Zoology, Elementary Physiology, Comparative Anatomy, Mammalian Physiology and Cellular Physiology and served as a research assistant for Dr. Dietz.

EXAMINATION AND THESIS REPORT

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Major Field: Physiology

Title of Thesis: Sodium Ion Transport Regulation by a Serotonin Stimulated Adenylate Cyclase System in Freshwater Mussels

Approved:

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